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THE UNIVERSITY OF ALBERTA

NUTRITIONAL FACTORS AFFECTING CLAVULANIC ACID PRODUCTION BY *STREPTOMYCES CLAVULIGERUS*.

BY

(C)

RICHARD ROBERT MAH

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR

THE DEGREE OF MASTER OF SCIENCE

DEPARTMENT OF MICROBIOLOGY

EDMONTON, ALBERTA

Fall, 1988

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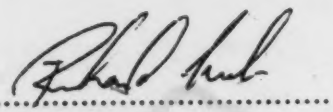

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The undersigned certify that they have read,

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Supervisor

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.....

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ABSTRACT

Streptomyces clavuligerus produces a number of beta lactam compounds such as penicillin N, desacetoxycephalosporin C, cephamycin C and clavulanic acid. Clavulanic acid has little antibacterial activity but is a powerful inhibitor of ß-lactamase enzymes. Previous studies have examined the nutritional effect of various carbon, nitrogen and phosphate sources on the production of penicillin and cephalosporin antibiotics. The nutritional effect on the production of clavulanic acid has been studied but the majority of the work has been in industry. Optimal production of clavulanic acid has been determined under complex nutrient conditions. Relatively few studies have been done examining clavulanic acid production under defined nutrient conditions as was done for the penicillins and cephalosporins. In this study, it was these same nutritional conditions that were examined for their effect on the production of clavulanic acid.

PREFACE

"I have become comfortably numb"

Pink Floyd. 'The Wall'
1979.

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I like to thank Dr. S.E. Jensen and my committee for their support and advice. I also wish to thank my family and friends for their encouragement during the dark periods.

Accolades must also go to modern technology for inventing the Macintosh and accessories, portable tape recorders, competitive table tennis and compact disc players. Without these, I would still be writing this thesis.

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ABBREVIATIONS

MIC	Minimum Inhibitory Concentration
sp.	species
PADAC	pyridine 2-azo-p-dimethylaniline
NTG	N-methyl-N'-nitro-N-nitrosoguanidine
MeOH	methanol
uv	ultraviolet
bioassay	biological assay
DCW	Dry Cell Weight
PM	production medium
PS	phytone seed

I. INTRODUCTION

Microbes produce a wide variety of compounds as a result of their biosynthetic activity, and these compounds can be broadly divided into two main groups, primary and secondary metabolites. Primary metabolites or tropholites, are essential for the survival of the organism. These are produced during the exponential phase (trophophase) of growth. The majority of the cells' energy is directed towards this process. Primary metabolism involves a series of interconnected enzyme mediated pathways. The catabolic, anabolic, and amphibolic pathways provide biosynthetic intermediates and energy and convert precursors into essential macromolecules such as deoxyribonucleic acid, ribonucleic acid, proteins, lipids, and polysaccharides (Martin and Demain, 1980). Metabolic intermediates rarely accumulate. Secondary metabolism occurs under specific nutritional conditions in certain taxonomic groups. Secondary metabolites or idiolites are produced after the exponential growth phase of the organism. This is the production phase also known as the idiophase. Idiolites tend to have unique chemical structures and are not essential for growth of the producing organism. Examples of these metabolites include steroids and antibiotics.

The biosynthetic pathways of the secondary metabolites are often connected to and influenced by the pathways of primary metabolism. An end product or an intermediate of a primary pathway often serves as a precursor for the secondary metabolites (Aharonowitz and Demain, 1978).

Secondary metabolite production is governed by two processes. The first is an overall regulatory control that operates as a function of growth rate. Idiolites are usually produced under conditions that promote suboptimal growth of the producing culture.

The second process affects individual biosynthetic pathways. Control is exerted by regulatory mechanisms such as induction, catabolite regulation and end product regulation (Martin and Demain, 1980).

Antibiotics make up one of the main classes of secondary metabolites. Production of the antibiotics usually occurs after exponential growth. In unicellular bacterial cells, there is a clear distinction between the exponential trophophase and the idiophase. With filamentous producing organisms such as actinomycetes and fungi, separation of the two phases is less distinct (Martin and Demain, 1980 and Brana *et al.*, 1986).

The production of ß-lactam antibiotics was first noticed in fungal genera of *Penicillium* in 1929 and *Cephalosporium* in 1953. Substances from each respective fungus were found to inhibit bacterial growth (Greenwood, 1982). These naturally produced penicillins and cephalosporins were the forerunners of the large assortment of ß-lactam antibiotics which play a major role in clinical treatment today.

In addition to the well known fungal producers, ß-lactam antibiotics have also been found to be produced by members of the Actinomycetes family, in particular the genus *Streptomyces*. In 1971, a new species, *S. clavuligerus*, was found to produce ß-lactam compounds such as penicillin N, desacetylcephalosporin C and cephamycin C (Nagarajan *et al.*, 1971). In 1976, this same species was found to produce a novel ß-lactam, clavulanic acid (Reading and Cole, 1977).

The biosynthetic pathways leading to penicillins and cephalosporins have been determined in both fungi and streptomycetes (Jensen *et al.*, 1982a and Balwin *et al.*, 1983a). Several enzymes from the biosynthetic pathways were isolated and partially or completely purified. Production of these ß-lactam compounds follows a similar route in

both of these groups of producers.

Clavulanic acid has a weak antibacterial activity spectrum but is a strong inhibitor of ß-lactamase type enzymes. The chemical structure of clavulanic acid is unique in that oxygen replaces sulfur in the 5 membered part of the bicyclic ring structure and it lacks the acylamino side chain on the ß-lactam nucleus which is characteristic of penicillins and cephalosporins. Although much work has been done on elucidating the origins of the atoms in the clavulanic acid molecule and on the development of complex nutrient media for optimal production, the biosynthetic pathway of clavulanic acid has yet to be determined.

The object of this study is to determine the nutritional requirements for the optimal production of clavulanic acid in a completely defined medium. Formulation of such a growth medium and elucidation of the nutritional factors which influence clavulanic acid production, form a basis for the subsequent investigation of the biosynthetic pathway leading to the isolation and identification of intermediates.

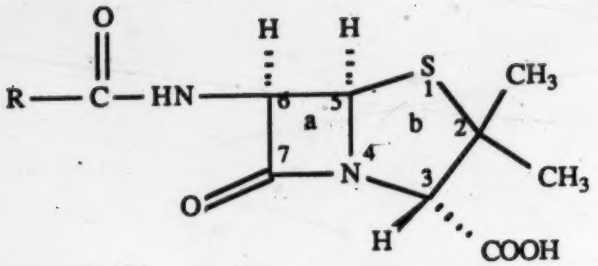
II. LITERATURE REVIEW

II.1. Overview of ß-lactams.

ß-lactam antibiotics were first reported to be produced, as fermentation products, in eukaryotic fungi; penicillin from *Penicillium* sp. and cephalosporin from *Cephalosporium*. sp. The discovery of penicillin has been well documented. First noticed by Alexander Fleming in 1929, penicillin was isolated and partially purified from *P. notatum* in the early 1940s (Greenwood, 1982). Further work aimed at increasing production of penicillin led to the use of higher yielding species, such as *P. chrysogenum*, and development of more efficient fermentation techniques (switch from surface cultures to submerged cultures). Although the penicillin isolated, penicillin G, was found to be effective, it had several limitations: a relatively narrow antimicrobial spectrum of activity, destruction by acidic environments, degradation by ß-lactamases produced by certain resistant bacteria and elicitation of allergic responses.

Penicillin, (as follows)

Penicillin



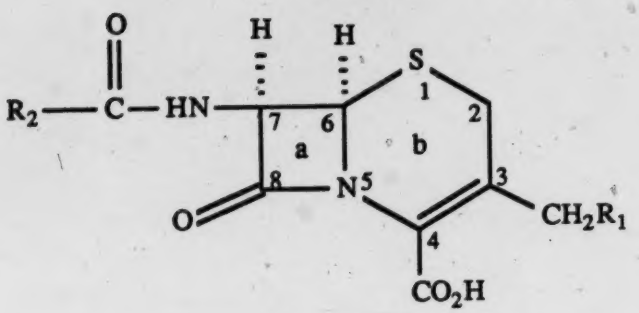
a=ß-lactam ring
b=thiazolidine ring
c=$C_6H_5CH_2$-, benzyl (pen G)

consists of two fused ring structures: a ß-lactam ring and a thiazolidine ring. At the 6 carbon position of the ß-lactam ring is the acylamino side chain. Modification of the side chain of penicillin circumvented some of the previously mentioned problems. Originally, the modification of the side chain was accomplished naturally through the incorporation of side chain precursors from the fermentation medium. Alternatively, 6-aminopenicillanic acid can be used as a starting material and chemically substituted with a wide range of side chains, much wider than the range which is accessible through the natural fermentation route. The penicillin nucleus, 6-aminopenicillanic acid, is a minor fermentation product. For the production of semisynthetic penicillins, 6-aminopenicillanic acid can be obtained by chemical hydrolysis or by the more efficient enzymic cleavage of the side chain of penicillin G with penicillin acylases (Abraham, 1984b). This allowed for more convenient modifications to produce a number of semisyntheic derivatives (Greenwood, 1982).

The new semisynthetic penicillins include two important classes: those resistant to ß-lactamases, and those with a broad antimicrobial spectrum of activity. Semisynthetic penicillins can be made resistant to ß-lactamases by addition of bulky side chain groups at the 6 amino position. Depending on the nature of the replacement side chains, the conformation of the active site of the ß-lactamase enzyme can be changed to render the enzyme catalytically inactive (Frere, 1985) or the size of the side chains can physically shield the ß-lactam ring from the ß-lactamase enzyme. The original penicillins produced by fermentation alone are primarily effective against Gram positive organisms. Replacement of the side chain with a positively charged moiety makes the penicillins effective against Gram negative organisms. The positive charge enhances penetration of the ß-lactam through the negatively charged lipopolysaccharide of the outer membrane of Gram negative organisms (Tipper, 1985). The disadvantage of the semisynthetic derivatives is that potency is generally reduced.

Cephalosporin C, a ß-lactam similar in structure to the penicillins,

Cephalosporin



a=ß-lactam ring
b=dihyhrothiazine ring

R1=-$OCOCH_3$
R2=$CO_2NH_2CH(CH_2)_3$- = Cephalosporin C

was discovered in 1953 (Abraham and Loder, 1972). Cephalosporins have several advantages over penicillins: the incidence of allergic responses upon treatment is lower, they have a broader antimicrobial spectrum of activity, and a higher resistance to ß-lactamases.

The structural difference between cephalosporins and penicillins is the replacement of the 5 membered thiazolidine ring of penicillin with a 6 membered dihydrothiazine ring of cephalosporin. Chemical modifications yielding semisynthetic cephalosporins are primarily made at two positions, carbon 3 and carbon 7 (Greenwood, 1982). As with the penicillins, the nucleus, 7-aminocephalosporanic acid, serves as the starting point for the production of semi-synthetic derivatives of cephalosporins. Unlike penicillins, side chain modification of cephalosporins can not be achieved through incorporation of alternative side chain precursors from the fermentation medium and an efficient

enzymatic process to convert cephalosporin C to 7-aminocephalosporanic acid is not available. Lower efficiency chemical deacylation is generally required (Bunnel *et al.*, 1986).

Chemical modifications are again used to improve the stability and activity of the cephalosporins. The original compounds produced by fermentation were poorly absorbed via oral administration and had to be injected in order to be effective. Chemical modification has been highly successful in yielding cephalosporins with improved antimicrobial activity, and moderately successful in improving stability, but most "third generation" cephalosporins must still be administered by injection (Greenwood, 1982).

Concurrent with the development of the ß-lactam fermentation in the fungi, Waksman began his pursuit of "soil microorganisms antagonistic to disease producing bacteria" (Waksman and Woodruff, 1940). Waksman concentrated his area of study within the actinomycetes. One of the first antagonistic substances discovered by Waksman and his coworkers was actinomycin. Subsequent studies led to the discovery of antibiotics such as streptothricin and streptomycin (Waksman, 1947b). Waksman also noted that the majority of the antagonistic actinomycetes belonged to three genera; *Norcardia, Micromonospora* and *Streptomyces* with most of the strains found in *Streptomyces*.

ß-lactam antibiotic production in *Streptomyces* has been noted as early as 1962 where one species was reported to yield penicillin N (Nagarajan *et al.*, 1971). Further studies led to the identification of penicillin N and the isolation and structural elucidation of a new group of ß-lactam antibiotics from *S. lipmanii* NRRL 3584 and a new species, *S. clavuligerus* NRRL 3585. The new ß-lactam compounds, cephamycins, resemble the

cephalosporins except for the replacement of the hydrogen with a methoxyl group at the 7 position of the cephalosporin nucleus (Nagarajan *et al.*, 1971). Both *S. lipmanii* and *S. clavuligerus*, were originally isolated from South American soil samples. The new species, *S. clavuligerus*, was named for the club like appendages that appear on the hyphae. In addition to ß-lactam compounds such as penicillin N, desacetylcephalosporin C and cephamycin C, *S. clavuligerus* was later found to produce a novel ß-lactam compound, clavulanic acid, which shows ß-lactamase inhibitory activity (Reading and Cole, 1977) .

II.2. Discovery of clavulanic acid.

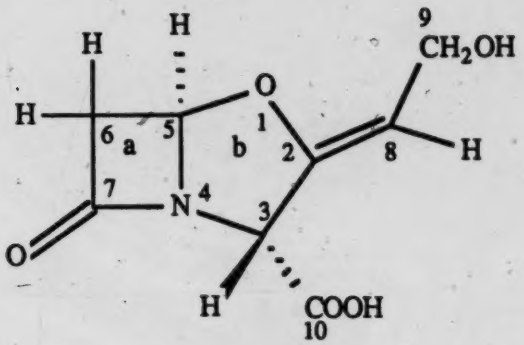
Clavulanic acid is a naturally occurring compound that was detected in culture filtrates from *S. clavuligerus*, during screening tests for ß-lactamase-inhibiting compounds. It became desirable to look for such compounds because of the increased incidence of penicillin and cephalosporin resistance due to production of ß-lactamases. Resistance to ß-lactamases can be improved by certain chemical modifications but an alternative approach was to search for compounds that would inhibit ß-lactamases, thereby (preventing hydrolysis of) protecting the susceptible ß-lactam (Sykes *et al.*, 1985). Early studies revealed some compounds that had inhibitory properties over a narrow range of β-lactamases. The importance of the discovery of clavulanic acid was its ability to inhibit a wide variety of ß-lactamases (Reading and Cole, 1977).

II.3. Clavulanic acid structure

Using X-ray crystallography, the structure of clavulanic acid was elucidated by Brown *et al.*, (1976) to be a fused bicyclic ß-lactam with the chemical nomenclature of

z-(2R,5R)-3-(ß-hydroxyethylidine)-7-oxo-4-oxa-1-azabicyclo-[3,2,0,]heptane-2-carboxylic acid.

Clavulanic acid



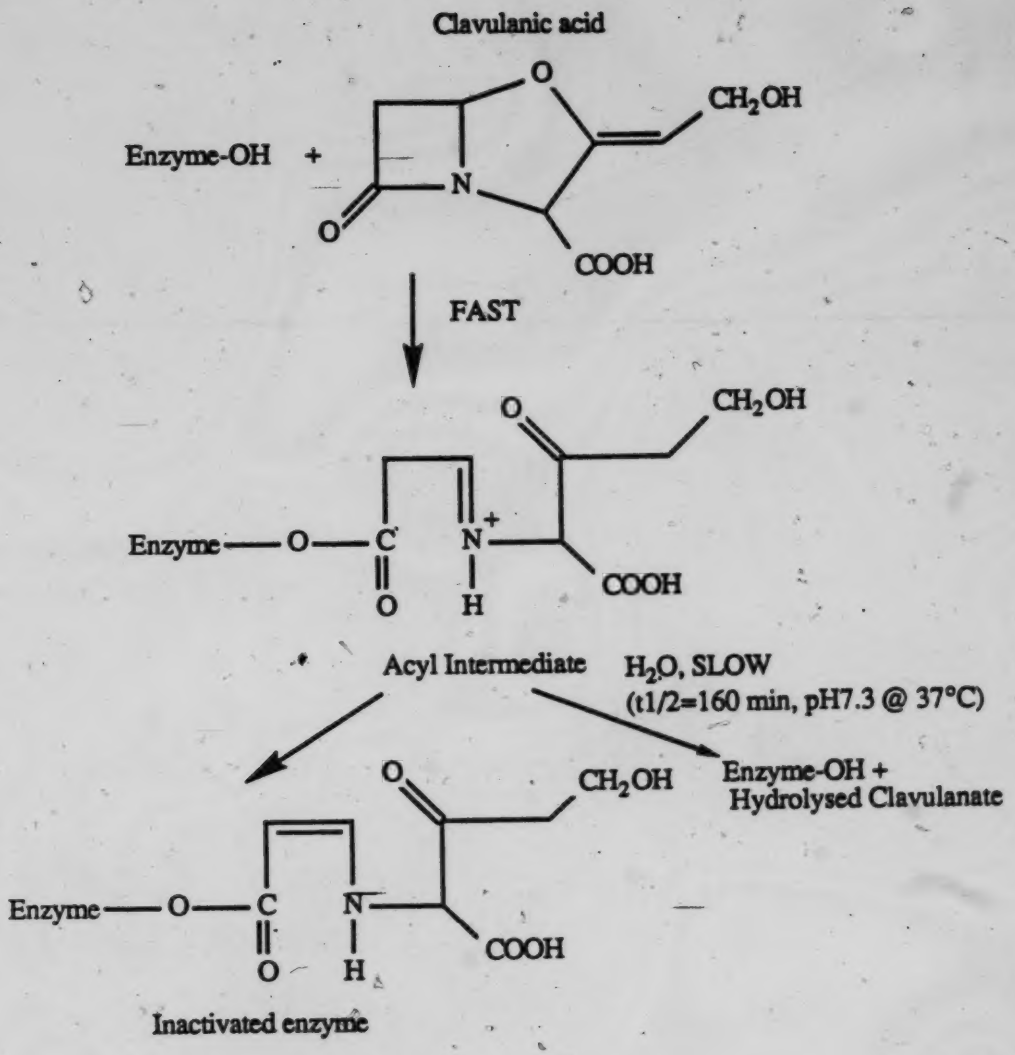
a=ß-lactam ring
b=oxazolidine ring

The structure superficially resembles the other ß-lactam compounds but there are several differences. Clavulanic acid lacks an acyl amino side chain at the carbon 6 position, a ß-hydroxyethylidine substituent is present at the carbon 2 position, and there is an oxygen molecule replacing the sulfur molecule found in both the thiazolidine ring of penicillin and dihydrothiazine ring in cephalosporin C (Reading and Cole, 1977). These differences suggest that the biosynthesis of clavulanic acid may occur in a separate pathway from that of penicillin and cephalosporin. Since the other ß-lactams are produced together with clavulanic acid, there may be mutual sharing of the biosynthetic intermediates.

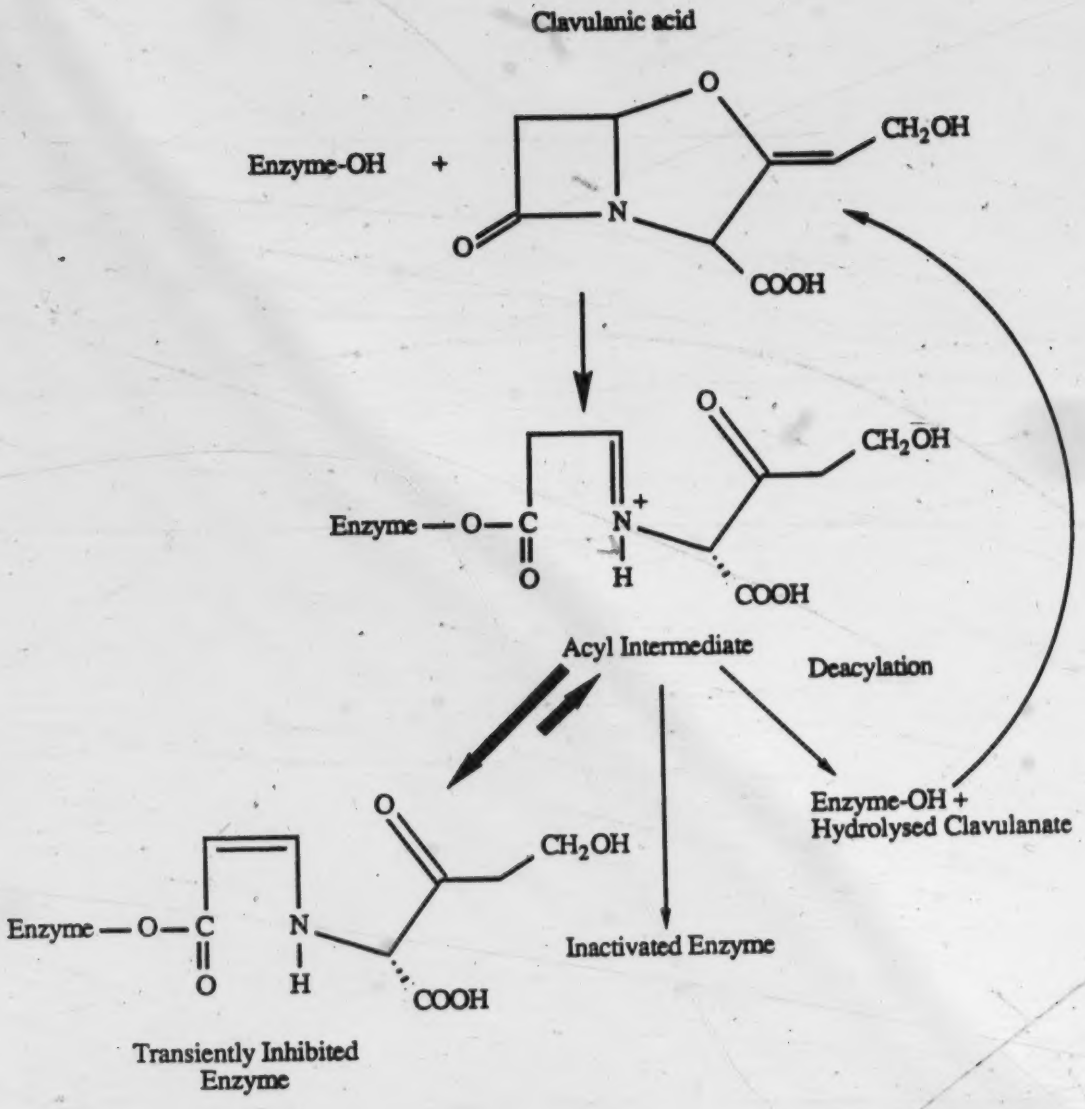
II.4. Properties of clavulanic acid

Clavulanic acid is a relatively weak broad-spectrum antibiotic with a minimum inhibitory concentration (MIC) ranging from 25-125 μg/ml (Reading and Cole, 1977). It acts synergistically with ß-lactamase sensitive penicillins and cephalosporins. The MIC's of these antibiotics against ß-lactamase producing resistant bacteria are lowered when used in conjunction with clavulanic acid. The synergy is the result of the ability of clavulanic acid to progressively inactivate the ß-lactamase therefore protecting the sensitive antibiotics from hydrolysis. The inhibitor is quite potent, and is effective against a wide range of ß-lactamases (Butterworth, 1984).

Clavulanic acid has a high affinity for the active site of a large number of ß-lactamases allowing it to compete efficiently with ß-lactam substrates. The ß-lactamase enzyme accepts clavulanic acid as the substrate and the peptide bond of the ß-lactam ring is cleaved to form an acyl intermediate. This is followed by the opening of the oxazolidine ring as diagrammed.


Clavulanic acid
Enzyme-OH +
O
CH2OH
N
O
COOH
FAST
CH2OH
O
Enzyme
O
C
N+
O
H
COOH
Acyl Intermediate
H2O, SLOW
(t1/2=160 min, pH7.3 @ 37°C)
Enzyme-OH +
Hydrolysed Clavulanate
O
CH2OH
Enzyme
O
C
N
O
H
COOH
Inactivated enzyme

These ring opening steps release reactive groups which form covalent bonds at the active site. In the case of a normal ß-lactam substrate, the enzyme acyl intermediate rapidly breaks down to release free active enzyme and hydrolysis products (Reading and Slocombe, 1986). With clavulanic acid, the clavulanate acyl intermediate is either relatively stable in its own right or achieves stability by further interaction with the enzyme. This is illustrated by the equilibrium between the acyl intermediate and the inactivated enzyme. Formation of the free enzyme and hydrolysed clavulanate from the acyl intermediate does occur but the reaction is slow. This sequence of events is characteristic of the inactivation of the staphylococcal ß-lactamase by clavulanic acid. The pathway of the reaction is linear and there is no detectable turnover, one molecule of clavulanic acid is required to inactivate one molecule of enzyme (Reading and Slocombe, 1986). Variations in this sequence of events occur with different types of ß-lactamases, for instance, the inactivation of the R-TEM ß-lactamase from *Escherichia coli* is more complex. The reaction follows a divergent pathway. The initial product is the same as the previously mentioned acyl intermediate, the ring opened clavulanate. The difference occurs after this stage as shown in the following diagram.


Clavulanic acid
Enzyme-OH +
O
CH2OH
N
O
COOH
Enzyme — O — C
O
N+
H
O
CH2OH
COOH
Acyl Intermediate
Deacylation
Enzyme-OH +
Hydrolysed Clavulanate
Inactivated Enzyme
Enzyme — O — C
O
N
H
O
CH2OH
COOH
Transiently Inhibited
Enzyme

From the acyl intermediate, three simultaneous reactions occur. One reaction releases free enzyme and hydrolytic products, resembling the case where a normal ß-lactam substrate is reacted with the ß-lactamase (Sykes and Bush 1982, Butterworth, 1984 and Reading and Slocombe, 1986). This loss of clavulanic acid accounts for the necessity of excess clavulanic acid to obtain complete inactivation of the enzyme. The other is the formation of the irreversibly inactivated enzyme complex. The irreversibly inactivated enzyme complex results from further covalent interaction between the ring-opened clavulanic acid and the enzyme. The third reaction is the formation of a transiently inhibited enzyme complex. This complex is the same as the one formed in the inactivation of the staphylococcal enzyme but it is only transiently stable. The transiently stable complex is formed at a faster rate than the irreversiblely inactivated enzyme complex. The transiently stable complex slowly releases free enzyme and hydrolytic products as a result of being in equilibrium with the initial acyl intermediate. Some of the enzyme will be irreversibly inactivated, but the majority of the enzyme is released in active form and reacts with fresh clavulanic acid to repeat the cycle. With each cycle, more enzyme is irreversibly inactivated until all the enzyme activity has been eliminated. This results in a requirement for more clavulanic acid to inhibit the *E. coli* ß-lactamase than the 1:1 stoichiometric reaction which is seen for the inactivated staphylococcal ß-lactamase (Reading and Slocombe, 1986). Estimates of the turnover number suggest that 115 clavulanic acid molecules are required per molecule of *E. coli* ß-lactamase (Sykes and Bush 1982, Butterworth, 1984 and Reading and Slocombe, 1986).

II.5. Regulation of antibiotic synthesis.

Antibiotics, like other secondary metabolites are not produced during the primary

growth stage of the culture. A number of different regulatory mechanisms have been been found to control antibiotic biosynthesis. With some antibiotics, accumulation of the antibiotic itself, in high concentrations, causes inhibition of antibiotic production. Examples of this include the production of chloramphenicol, penicillin and mycophenolic acid (Elander and Aoki, 1982).

Components of primary metabolic pathways can also play a major role in the regulation of antibiotic synthesis. An example of such an influence is the production of lysine which affects penicillin production. Lysine, as an end product of the branched lysine biosynthetic pathway, exerts feedback inhibition on its own production in *P. chrysogenum*. Since one of the precursor amino acids for penicillin, α-aminoadipate, also arises from this pathway, lysine levels also affect penicillin production. The lysine biosynthetic pathway branches at α-aminoadipate, the common intermediate of lysine and penicillin. When primary metabolic feedback inhibition by lysine occurs, the enzymes responsible for α-aminoadipate production are inhibited, resulting in decreased production of both lysine and penicillin (Martin and Demain, 1980).

Another type of regulation which is frequently observed to control antibiotic production is carbon catabolite regulation. In this regulatory process, the biosynthesis of antibiotics is regulated by catabolites of rapidly used carbon sources. An example of this is the glucose regulation of penicillin production by *P. chrysogenum*. Glucose is a substrate more suited for growth than for antibiotic production. Low levels of antibiotics are produced when glucose serves as the carbon source. Polysaccharides or oligosaccharides are more slowly utilized than glucose and therfore are better carbon

sources for antibiotic production (Martin and Demain, 1980). Usually a penicillin production medium employs a combination of both glucose and a more slowly utilizable carbon source such as lactose. Glucose is rapidly utilized in the trophophase and allows the culture to become well established. After glucose exhaustion, lactose is utilized for penicillin production.

Recently, the biosynthetic pathway of penicillin and cephalosporin production in *S. clavuligerus* has been elucidated (Jensen *et al.*, 1982a and 1982b). Nutritional requirements and factors regulating penicillin and cephalosporin production by this organism have also been determined (Aharonowitz and Demain, 1978, 1979). For optimum production of cephalosporins, the best carbon sources were determined to be starch, starch hydrolysates, glycerol and maltose. Organic acids such as α-ketoglutarate and succinate were also able to support antibiotic production but not to the same level as the carbohydrates. Antibiotic production was found to be affected by the availability of the carbon source. Increasing the amounts of the preferred carbon source decreased the production of the cephalosporins. An example of this is the ability of *S. clavuligerus* to produce maximum levels of cephalosporin with 1% glycerol although maximum growth yields were not obtained until 5% glycerol was provided (Aharonowitz and Demain, 1978). Poorly utilized carbon sources such as α-ketoglutarate led to higher specific production (μg/mg cell dry weight) and shifted the fermentation to be more closely associated with growth (Aharonowitz and Demain, 1979). Total antibiotic production (μg/ml) was, however, lower than with carbohydrate carbon sources such as starch.

Production of cephalosporins by *S. clavuligerus* was also affected by the type of

nitrogen source. Asparagine, glutamine and arginine best suited the organism as nitrogen sources for antibiotic production. Production levels were slightly lower with urea as the nitrogen source. Inorganic nitrogen in the form of NH_4Cl supported growth but low levels of antibiotics were produced. Addition of NH_4^+ to medium containing asparagine as the primary nitrogen source, increased the amount of growth (mycelial mass) but reduced the production of antibiotics by 75% (Aharonowitz, 1979).

Phosphate concentration also influenced the levels of cephalosporin production. The best product yields were obtained at phosphate concentrations that were suboptimal for vegetative growth (Aharonowitz and Demain, 1977). Experiments with resting cell cultures showed that the negative effect caused by the phosphate was caused by both the inhibition of activity and repression of production of the enzymes required for antibiotic production (Lubbe *et al.*, 1985b).

II.6. Biosynthesis of clavulanic acid

Since the first description of clavulanic acid and its production by *S. clavuligerus*, there have been very few reports of its production by other organisms (Butterworth, 1984). Three other species of *Streptomyces*, *S. jumonjinensis*, *S. katsurahamanus*, and *Streptomyces* sp. P6621 have been reported to produce clavulanic acid. To date, the majority of the work done on clavulanic acid production has been reported for *S. clavuligerus*. Not much information is available about production in the other species other than their intial description in the patent literature (Butterworth, *et al.*, 1983 and Butterworth, 1984).

Because clavulanic acid is a ß-lactam compound and *S. clavuligerus* coproduces

other ß-lactam antibiotics together with clavulanic acid, there was some suggestion that clavulanic acid might undergo a biosynthetic process similar to the one that produces penicillins, cephamycins and cephalosporins (Elson and Oliver, 1978). These latter antibiotics are formed from the amino acids, L-α-aminoadipic acid, L-cysteine and L-valine, via a tripeptide, δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV). The tripeptide undergoes cyclization, epimerization and ring expansion to form penicillins and cephalosporins (Jensen *et al.*, 1982b). Closer inspection of the clavulanic acid structure however, shows more differences than similarities to the above mentioned penicillins and cephalosporins. Oxygen replaces sulfur in the oxazoloidine ring and the inhibitor lacks an aminoacyl side chain as well as a 6-amino group (Reading and Cole, 1977). These differences suggest that clavulanate arises from a separate biosynthetic pathway although it may have some common steps or intermediates with the other pathway (Elson and Oliver, 1978).

Several hypotheses on the origin of the clavulanic acid structure have been formulated from feeding studies using radioactively labelled precursors. One possible precursor of the three carbon atom in the ß-lactam ring structure in clavulanic acid was thought to be propionic acid. From the experiments of Elson and Oliver in which doubly labelled acetate and glycerol, and labelled bicarbonate and propionic acid were incorporated into the fermentation medium, the carbon skeleton of the ß-lactam ring of clavulanic acid was found not to be derived directly from propionate but from a 3 carbon intermediate of glycolysis (Elson and Oliver, 1978). Subsequent study has strongly implicated D-glycerate as the C_3 intermediate required in the formation of the ß-lactam

ring (Townsend and Ho, 1985b). Another possible precursor for the ß-lactam ring is ß-hydroxypropionic acid. High retention of the radioactive atoms in the clavulanic acid structure correlated to an intact incorporation of ß-hydroxypropionic acid (Gutman *et al.*, 1985a).

The biosynthetic origin of the five remaining carbon atoms of clavulanic acid has also been investigated in a similar manner by following the incorporation of radioactively labelled compounds. These studies indicated that the five remaining carbon atoms appear to be derived from α-ketoglutarate which probably was derived, in turn, from glutamate (Elson and Oliver, 1981). One possible precursor, ACV, was ruled out on the basis that the incorporation pattern of labelled valine was different from the observed labelling pattern in clavulanic acid (Elson and Oliver, 1978). A more recent study presented evidence that the urea cycle amino acids, particularly ornithine, are incorporated at 15-20 times higher efficiency than glutamic acid (Townsend and Ho, 1985a). Romero *et al.* also found evidence that these urea cycle amino acids were better precursors than glutamic acid, and that glutamic acid actually depressed the production of clavulanic acid (Romero *et al.*, 1984). The oxidation state of the γ-carboxyl group of glutamic acid is higher than the γ-hydroxy group in the C9 position clavulanic acid. This indicated that a reduction step would be required before incorporation of glutamate into clavulanic acid.

The basic scheme for the biosynthesis of clavulanic acid which has emerged from these studies is the coupling of the carbon skeleton which will eventually form the ß-lactam ring, probably from glycerate or ß-hydroxypropionic acid, with a C_5 amino

acid or amino acid derivative such as ornithine, to yield a linear dipeptide-like precursor of clavulanic acid (Gutman *et al.*, 1985a).

II.7. Production of Clavulanic Acid

S. clavuligerus produces clavulanic acid as a fermentation product when it is cultivated in a suitable chemically defined or complex nutrient medium with adequate aeration (Cole *et al.*, 1985). Clavulanic acid is totally excreted into the medium by the cells. The free acid form of clavulanic acid is quite unstable, but sodium or potassium salts of clavulanic acid, are more stable, as is the ester form (Reading and Cole, 1977 and Cole *et al.*, 1985). Industrial fermentation processes extract clavulanic acid from the culture medium in the salt form.

Nutritional studies similar to those that were done on the production of cephalosporins by *S. clavuligerus*, have been conducted to investigate clavulanic acid production, but much less information from studies using defined media is available. The majority of the research on clavulanic acid production has been carried out by industry, and so the published literature may only represent a part of the total work which has been done. The producing organism requires a suitable source of carbon, nitrogen, phosphate and mineral salts. For production of clavulanic acid, the suggested carbohydrate content of the growth medium varies from 0.1-5% (v/v) depending on the particular carbon source used (Cole *et al.*, 1985). Recommended carbon sources included both defined and undefined carbohydrates. The best defined carbon sources include starch, starch hydrolysate, dextrin, sucrose, lactose or other sugars and glycerol or glycerol esters. Glucose was mentioned as a carbon source which supported good growth but was undesirable in some media as it depressed clavulanic acid production. In

contrast, it was reported by Higgins and Kastner (1971) that glucose cannot be utilized by *S. clavuligerus* and this finding has been confirmed by other workers (Aharonowitz and Demain, 1977). Carboxylic acids, derivatives of animal fats, vegetable oils, peptones and beef extracts can also be used for growth and production of clavulanic acid.

Recommended nitrogen sources for clavulanic acid production are generally used in amounts ranging from 0.1-10% (Cole *et al.*, 1985). For complex nutrient media, yeast extract, corn steep liquor, vegetable protein, fish and meat extracts and peptones are suitable nitrogen sources. Defined media make use of chemically defined nitrogen sources such as urea, NH_4Cl, amides and amino acids (valine, asparagine, glutamic acid, glutamine, proline and phenylalanine) used singly or in combinations (Cole *et al.*, 1985). In one study, the production medium was buffered with 3-[N-morpholino] propanesulfonic acid (MOPS) to avoid any possible inhibitory/repressive effect of inorganic phosphate (Lubbe *et al.*, 1985b).

In the U.S. patent by Cole *et al.* (1985), 14 different types of complex nutrient media were used as production medium for production of clavulanic acid by *S. clavuligerus*. The amount of clavulanic acid produced was reported as a percentage of ß-lactamase inhibition activity which allowed comparisons to be made between the various media but no absolute production values were presented. In these studies, the optimal production of clavulanic acid occurred 3-5 days into the fermentation. In one case, the production of clavulanic acid in shake flask cultures was compared to production in a fermenter and actual production levels were given. The shake flask

cultures produced 300-500μg/ml. In comparison, the fermenter culture growing in the same production medium, produced lower amounts ranging from 200-300 μg/ml (Cole *et al.*, 1985).

II. 8. Regulation of clavulanic acid production.

The regulation of the production of clavulanic acid in a defined system has been studied by Romero *et al.*, (1984). They looked at the effect of carbon, nitrogen and phosphate levels on production and found that glycerol was the most important carbon source that affected clavulanic acid production. In the absence of glycerol, there was no formation of the inhibitor. As glycerol was depleted from the growth medium, production of clavulanic acid decreased as well. Maximum production of clavulanic acid was obtained with 110mM (1% w/v) glycerol present in the production medium. Concentrations above this reduced production levels of clavulanic acid (Romero *et al.*, 1984). A separate study by Ho in 1985, indicated that the same amount of clavulanic acid can be produced with glycerol at 3.5% (w/v) (380mM) but this medium also contained complex carbon sources in the form of soybean flour (Ho, 1985).

When the effect of nitrogen source was investigated, Romero *et al.* found that additional feeding of nitrogen in the form of glutamic acid or NH_4Cl to production medium already supplemented with 10 or 20 mM glutamic acid, drastically reduced clavulanic acid production levels. They also determined that there is an concentration dependent inhibitory effect by phosphate. Addition of phosphate past the optimum of 25 mM, reduced the clavulanic acid production (Romero *et al.*, 1984).

II. 9. CLAVULANIC ACID ASSAYS

Several assay systems have been described for qualitative and quantitative analysis of clavulanic acid in culture filtrates. One such quantitative method is the chemical assay as described by Bird *et al.* and Foulstone and Reading (Bird *et al.*, 1982 and Foulstone and Reading, 1982).

Clavulanic acid lacks a chromophore, making spectrophotometric detection difficult. In the procedure described by both groups (Bird *et al.*, 1982 and Foulstone and Reading, 1982), this problem is solved by complexing imidazole with clavulanic acid to give a derivative with a detectable chromophore. Reacting imidazole with clavulanic acid forms the derivative, 1-(4-aza-8-hydroxy-6-oxo) oct-2-en-1-oylimidazole which can be detected by its absorbance at a wavelength of 311nm. The disadvantage of using 311nm as the wavelength of detection is that other components in the culture filtrates also absorb at this wavelength thereby masking the absorbance maximum of the imidazole-derivatized clavulanic acid.

Another method of analysis relies on the separation of the clavulanic acid from the medium components by high performance liquid chromatography. Clavulanic acid is resolved from the other components by chromatography on reverse phase C-18 type columns. Separation is based on the hydrophilicity or hydrophobicity of the various compounds to be resolved and separation can be altered by manipulation of the pH and composition of the mobile phase (Harris and Kratochvil, 1980).

Hydrophilic compounds such as clavulanic acid are usually poorly retained in such chromatography systems and may not be resolved from other medium components. Retention of charged compounds can be improved by incorporation of an ion pairing

agent into the mobile phase. These reagents contain large hydrophobic counter ions which complex with positive or negatively charged compounds to form neutral ion pairs that act as nonpolar solutes in the mobile phase. Clavulanic acid is negatively charged at neutral pH and will form ion pairs with tetrabutyl ammonium salts (Pic A reagent, Waters Scientific Co.).

Once separated from the other medium components, clavulanic acid can be detected at 214nm. Alternatively, a pre-column derivation with imidazole, as described for the chemical assay, can be used to solve both retention and detection problems since the imidazole derivative of clavulanic acid can be detected by measuring absorption at 311nm (Bird *et al.*, 1982 and Foulstone and Reading, 1982).

A third type of method for analyzing clavulanic acid content of culture filtrates uses an enzymatic assay which measures ß-lactamase activity. Detection in ß-lactamase enzyme assays is based on the ability of clavulanic acid to inhibit the degradative action of the ß-lactamase and therefore leave the ß-lactam substrate intact. Quantitation can be made by comparing the rate of degradation of the ß-lactam substrate in the presence and absence of known amounts of clavulanic acid. Several types of ß-lactam substrates have been used in these systems including; pyridine-2-azo-p-dimethylaniline cephalosporin (PADAC) and penicillin G.

Pyridine-2-azo-p-dimethylaniline (PADAC) is a coloured cephalosporin substrate that undergoes a colour change when the ß-lactam ring is cleaved. The chromophore, pyridine-2-azo-p-dimethylaniline, is released from the structure when the ß-lactam ring is cleaved, resulting in a colour change from purple to yellow (Jones *et al.*, 1982). This color change can be followed spectrophotometrically by measuring the decrease in

absorbance at 572nm (Schindler and Huber, 1980). A similar colorimetric assay uses nitrocefin as the substrate. Nitrocefin is a weakly active cephalosporin that changes its colour from yellow to red when the amide bond of the ß-lactam ring is hydrolysed (Uri *et al.*, 1978).

The related method of Reading and Hepburn (1979) and Gutman *et al.* (1985b) monitors the degradation of penicillin G by following the change in absorbance at 240nm. The molar absorption coefficient of penicillin G at 240nm, is greater than that of its corresponding degradation product, penicilloic acid (Gutman *et al.*, 1985). Upon addition of the ß-lactamase, absorbance at 240nm will decrease. In the presence of clavulanic acid, the rate of decrease of absorbance will be less rapid. Both of these enzymatic assays are rapid and sensitive and are less subject to interference by medium components than is the chemical assay.

Agar diffusion biological assays have also been described to measure clavulanic acid content of culture filtrates (Reading and Cole, 1977) but these assays are best used in a qualitative manner. These assays make use of the inhibitory properties of clavulanic acid on ß-lactamase enzymes produced by ß-lactam resistant indicator organisms. Typically, a penicillin G resistant indicator organism such as *Staphylococcus aureus* or *Klebsiella pneumoniae* is cultivated in penicillin G supplemented solid medium. The ß-lactamase produced by the indicator organism destroys penicillin G allowing for growth in the medium. The addition of clavulanic acid on filter paper discs inhibits the ß-lactamase allowing penicillin G to lyse the indicator organism. This is indicated by clear zones around the filter disc (zones of inhibition). The diameter of the zone of inhibition is related to the logarithm of the amount of clavulanic acid applied. This relationship

allows rough quantitiation of the clavulanic acid content but the assay is subject to many sources of variability including the physiological state of the indicator organism, incubation temperature and humidity and the surface to volume ratio of the agar medium.

The purpose of this study is to apply the same types of nutritional studies to the production of clavulanic acid using the same production medium that was developed by Aharonowitz for studying cephalosporin production (Aharonowitz and Demain, 1977). Although Romero *et al.* (1984) conducted similar studies on clavulanic acid production using a defined system, they used a production medium that contained several components which could serve as both carbon and nitrogen sources, as well as mixtures of more than one carbon and nitrogen source. In contrast, the production medium devised by Aharonowitz for the study of cephalosporin production has only one carbon and nitrogen source making it easier to determine which components specifically affect the production of clavulanic acid. Once defined nutritional conditions supporting maximum production of clavulanic acid have been determined, manipulations to the growth medium constituents may cause an inhibition of clavulanic acid biosynthesis which could result in accumulation of biosynthetic intermediates. Identification of such intermediates would help in understanding the biosynthetic pathway of clavulanic acid.

III. MATERIALS AND METHODS

III.1. CULTURES

The microbial cultures used in this investigation were:

Streptomyces clavuligerus NRRL 3585,

Streptomyces clavuligerus strain NTG 12,

Staphylococcus aureus N2.

S. clavuligerus NRRL 3585 is a wild type culture originally obtained from the Northern Regional Research Laboratory, Peoria, Illinois. *S. clavuligerus* strain NTG 12 is a mutant strain of *S. clavuligerus* which does not produce penicillin or cephalosporin type antibiotics. It was isolated from the wild type strain after mutagenesis with N-methyl-N'-nitro-N nitrosoguanidine, and provided by Dr. S. E. Jensen. *S. aureus* N2 is a penicillin resistant strain obtained from our Departmental culture collection. The penicillin resistance is due to the presence of a ß-lactamase type enzyme.

III.2. MAINTENANCE OF CULTURES

A. All *S. clavuligerus* cultures were stored as spore suspension in 20% (v/v) glycerol solutions at -75° C. Spores were obtained by streaking *S. clavuligerus* cultures onto tomato oatmeal agar TOA plates of the following composition:

Tomato Oatmeal Agar

oatmeal pablum	8 g
tomato paste	8 g
Bacto agar (Difco)	10 g

Final volume 400 ml; pH was adjusted to 6.8 before sterilization by autoclaving for 1 h.

Streaked plates were incubated at 28°C for 1-2 weeks until abundant blue-gray spores had developed. The spores were scraped off and collected into a sterile 20% (v/v) glycerol solution (0.5 ml per agar plate). Spores were dispersed in the glycerol solution by brief (1-5 minutes) sonication in a Bransonic 42 sonic bath. The spore suspensions were dispensed in 0.5 ml amounts in sterile screw cap plastic tubes (Nunc brand) and stored at -75°C. The spore content of the stocks was determined by a plate count on a randomly chosen sample. The sample was serially diluted and spread onto phytone seed agar (PSA) of the following composition:

Phytone Seed Agar

glycerol	1.0g
sucrose	2.0g
tryptone	0.5g
phytone	1.5g
K_2HPO_4	0.02g
Bacto agar (Difco)	2.0g

final volume 100ml; pH was adjusted to 6.8 before autoclaving for 15 min. The plates were incubated at 28°C for 24 h before resulting colonies were counted. The spore stocks used throughout this study contained about 10^8- 10^9 viable spores/ml.

B. *S. aureus* N2 was grown and maintained in trypticase soy broth (TCS) containing 30g of trypticase soy broth (BBL) per litre supplemented with penicillin G to a final concentration of 1 μg/ml and with 20% (v/v) glycerol. Penicillin G was added as a filter sterilized stock solution to the TCS broth after autoclaving. Penicillin G is included in the medium to induce ß-lactamase enzyme production. Cultures were incubated overnight at 37°C. The cultures were stored at -75°C in two ml aliquots.

III.3. NUTRITIONAL STUDIES

Studies conducted to determine the effects of growth medium on the production of clavulanic acid were carried out in a defined growth medium originally described by Aharonowitz *et al.*, (Aharonowitz *et al.*, 1977). This basal production medium (PM) contained:

Production medium

glycerol	10.0 g
asparagine	2.0 g
MOPS buffer	21.0 g
K_2HPO_4	4.4 g
$MgSO_4 \cdot 7\ H_2O$	0.6 g
$FeSO_4 \cdot 7\ H_2O$	1.0 mg
$MnCl_2 \cdot 4\ H_2O$	1.0 mg
$ZnSO_4 \cdot 3\ H_2O$	1.0 mg
$CaCl_2 \cdot 2\ H_20$	13.0 mg

final volume 1L; pH was adjusted to 6.8 before autoclaving for 15 min.

The effects of carbon sources and nitrogen sources on clavulanic acid production were tested by replacing glycerol and/or asparagine respectively with various amounts and types of carbon and nitrogen containing compounds. When amino acids were used as growth medium components, they were filtered sterilized and added separately after sterilization. The effect of phosphate on clavulanic acid production was tested by varying the amount of inorganic phosphate.

III.4. INOCULUM PREPARATION.

Culture uniformity was optimized by using a two stage inoculum preparation. In the

first stage, spore stocks of *S. clavuligerus* were transferred to phytone seed (PS) medium (0.5ml/spore stock per 25ml of PS medium in 125ml erlenmeyer flask) for germination. The spores were incubated at 28°C on a New Brunswick shaker at 250 rpm. Maximum vegetative growth occurred at about 48h.

The germinated cells were harvested asectically by filtration through a pre-wetted Whatman #2 filter, washed with sterile distilled H_2O, scraped off and resuspended in distilled H_2O to the original culture volume.

In the second stage, the inoculation of the production medium was achieved by adding the washed inoculum to double strength production medium to a final concentration of 4% (v/v). The mixture was dispensed in 50 ml aliquots into sterile 500ml erlenmeyer flasks containing 50ml of distilled H_2O. When individual components of the production medium were to be varied, double strength production medium was prepared lacking the component to be examined. The inoculated double strength production medium was then dispensed as described as above into 500 ml flasks containing 50 ml of appropiate solutions of the growth medium component to be examined. Triplicate cultures for each growth medium composition were incubated on a New Brunswick platform shaker at 250 rpm at 28°C. Samples were taken every 24 hours. The distilled H_2O used for inoculum washing and medium preparation was prepared by filtration through a Milli-Q filtration system (Millipore Ltd) and was sterilized by autoclaving.

III.5. ANALYSIS

Dry Cell Weight Determination and Sampling.

Ten ml of the culture was taken from each flask. The cells were harvested from the production medium by vacuum filtration through millipore filters (HA 0.45 µm Millipore) for dry cell weight determination. The filtered cells were washed with distilled water and dried in a dessicator for 24 h before weighing.

III.6. CLAVULANIC ACID DETERMINATION

A number of different methods of measuring clavulanic acid production in fermentation media were evaluated.

A. Qualitative Analysis

A qualitative agar diffusion assay was included as an independent method to check the results that were reported by the other methods. The biological assay (bioassay) makes use of the inhibitory properties of clavulanic acid on ß-lactamase enzymes produced by ß-lactam resistant indicator organisms. A penicillin G resistant strain of *S. aureus* was used as the indicator organism in the bioassay. One ml of *S. aureus* was added to penicillin G supplemented (final concentration of 1µg/ml) TCS agar. Ten millilitres of TCS agar were dispensed per petri plate. Twenty microlitres of filtrates or standards with clavulanic acid were spotted onto 6.35 mm diameter paper discs, one disc per plate. The range of clavulanic acid was 0.0-2.0µg. The plates were incubated at 37° C overnight. Inhibition was indicated by clear zones around the filter disc (zones of inhibition). Rough quantiation was done by correlating the zone size with the logarithm of the amount of clavulanic acid applied.

B. Quantitative Analysis.

1. Chemical Assay.

One method of determining the clavulanic acid content of culture filtrates production used the chemical assay that was described by Bird *et al.*, and Foulstone and Reading (Bird *et al.*, 1982 and Foulstone and Reading, 1982). In this chemical assay, imidazole was complexed with clavulanic acid to produce a spectrophotometrically detectable chromophore with absorbance maximum at 311nm.

The imidazole-derivatized clavulanic acid was formed by reacting 0.25 mL of imidazole reagent with 1mL of sample solution at ambient temperatures (25° C). The imidazole used was obtained from the Sigma Chemical Co. St. Louis MO. with a grade III low fluorescence blank rating. The imidazole reagent was prepared by dissolving 8.25 g of imidazole in 24ml of distilled water plus 2 ml of 5M HCl. The solution was adjusted to pH 6.8 by the addition of 5 M HCl, and the volume was at 45 ml. The product was determined to be stable for 2-3 hours at ambient temperatures. The reagent had a shelf life of two months at ambient temperatures (25° C).

2. High Performance Liquid Chromatography (HPLC) Assays.

High pressure liquid chromatography was carried out using a model M-45 pump, model 680 automated gradient controller, model UK-6 injector, and model 480 variable wavelength UV detector all from Waters Scientific Co. Data were recorded on a Hewlett-Packard model 3390 A integrator.

a) Underivatized Clavulanic Acid. In initial studies, culture filtrates were analyzed without any modifications of the sample.

i) Standard mobile phase. Culture filtrates were analyzed using a RP-18 C-18 phase column (10 µm sphere diameter, Brownlee Labs) to separate the clavulanic acid from the medium components. The mobile phase used was: 50mM KH_2PO_4 in 10% methanol (adjusted to pH 3.2 with H_3PO_4).

Samples were analyzed at a flow rate of 2ml/min and detection was at 214nm.

ii) Modified Mobile Phase. Retention of clavulanic acid was enhanced by modification of the HPLC system described above to incorporate an ion pairing agent into the mobile phase. The new mobile phase was: methanol-10%, 90% H_20 containing PIC A reagent at 3ml/100ml (Waters Scientific Co.). All other conditions remained the same.

b) Derivatized Clavulanic acid.

Resolution and separation was best acheived with a pre-column derivation of the clavulanic acid with imidazole. Derivation of the sample was the same as described in the chemical assay. The stationary phase used was a C-18 reverse phase µ-Bonda pak column compressed in a Waters Z module radial compression chamber. The mobile phase used was: 0.1M KH_2PO_4-94%; MeOH- 6% at pH 3.2 (Foulstone and Reading, 1982) with a flow rate of 2 ml/min. Detection was at 311nm.

3. Enzyme assays.

Clavulanic acid content was also measured using ß-lactamase enzyme assays. The enzyme assays are based on the ability of clavulanic acid to inhibit the degradative activity of ß-lactamase. Quantitation was made by comparing the rate of degradation of the ß-lactam substrate in the presence and absence of known amounts of clavulanic acid. The addition of clavulanic acid decreased the rate of degradation of the ß-lactams by the ß-lactamases. A standard curve was obtained which relates the amount of clavulanic acid to the degree of ß-lactamase inhibition, expressed as a percentage of the uninhibited control (Gutman *et al.*, 1985). Two different ß-lactam substrates were tested; pyridine-2-azo-p-dimethylaniline cephalosporin (PADAC) and penicillin G.

a. PADAC is a coloured cephalosporin substrate that undergoes a colour change when the ß-lactam ring is cleaved. The reaction was monitored spectrophotometrically by measuring the decrease in absorbance at 572nm (Schindler and Huber, 1980).

In the assay, 100,000 units of penicillinase (Difco type I) was combined with 2.5 µg of clavulanic acid standard in a final volume of 0.02ml of 0.1M sodium phosphate buffer, pH 7.0. The mixture was incubated for 5 minutes at 30°C in a Julabo model VL F10 water bath. Nine hundred and eighty microlitres of 3.96 µg/ml PADAC buffered at pH 7.0 was added to the inhibitor-enzyme mixture. The reaction was mixed and the rate of degradation was followed at 570nm with a model SP 8-100 Pye Unicam UV recording spectrophotometer. When the filtrates were analyzed, 10 µl was substituted for the clavulanic acid standard.

b. Penicillin G-Difco penicillinase type I

The method of Reading and Hepburn, and Gutman *et al.*, (Reading and Hepburn, 1979, and Gutman *et al.*, 1985) monitored the degradation of penicillin G at an absorbance of 240nm. Fifteen thousand units of penicillinase (Difco type I) was combined with 0 - 10 µg of clavulanic acid in a final volume of 0.5 ml of 0.1M sodium phosphate buffer, pH 6.8. The mixture was preincubated for 5 minutes at 37°C in a Julabo model VL F10 water bath. After the preincubation, 500 µL of 300 µg/ml penicillin G was added to the mixture and the rate of degradation was followed at 240nm using the previously mentioned spectrophotometer. When the filtrates were analyzed, 25 µl was combined with the enzyme and the above mentioned procedure was followed.

IV. RESULTS

The nutritional studies carried out in this investigation required the development of reliable methods for measuring the clavulanic acid content of culture filtrates. Several different methods of quantitation of clavulanic acid content were investigated including biological, chemical, HPLC and enzymatic assays.

IV.1. CLAVULANIC ACID ANALYSES

A. Biological Assay (Bioassay) of Clavulanic Acid.

This agar diffusion assay utilizes an indicator organism to measure clavulanic acid content of culture filtrates (Reading and Cole, 1977). *S. aureus* N2 was used as the penicillin G resistant, ß-lactamase producing indicator organism. A linear reponse standard curve was obtained when the inhibition zone size was plotted against the logarithm of clavulanic acid concentration (figure 1). Since the size of the zones varied from one bioassay to the next, a set a standards was included each time a bioassay was done. Despite its limitations, the bioassay was found to be a good qualitative tool for detecting clavulanic acid production.

B.1. Chemical Assay of Clavulanic Acid Standards.

The chemical assay method of Bird *et al.* (1982) and Foulstone and Reading (1982), was used to set up a clavulanic acid standard curve. Various amounts of clavulanic acid standards were derivatized by the reaction with imidazole reagent as described in Materials and Methods. A linear relationship between A_{312}nm and concentration was observed from 0 to 30 µg of clavulanic acid (Figure 2). Similar results were obtained when clavulanic acid standards were prepared in 0.1M MOPS buffer pH 6.8, distilled

Figure 1. Standard curve of clavulanic acid as determined by the bioassay method. Twenty microlitre amounts of clavulanic acid standards were spotted onto paper discs placed on TCS agar supplemented with penicillin G to a final concentration of 1 μg/ml and inoculated with *S. aureus* N2. Plates were incubated overnight at 37°C and then zones of inhibition were measured.

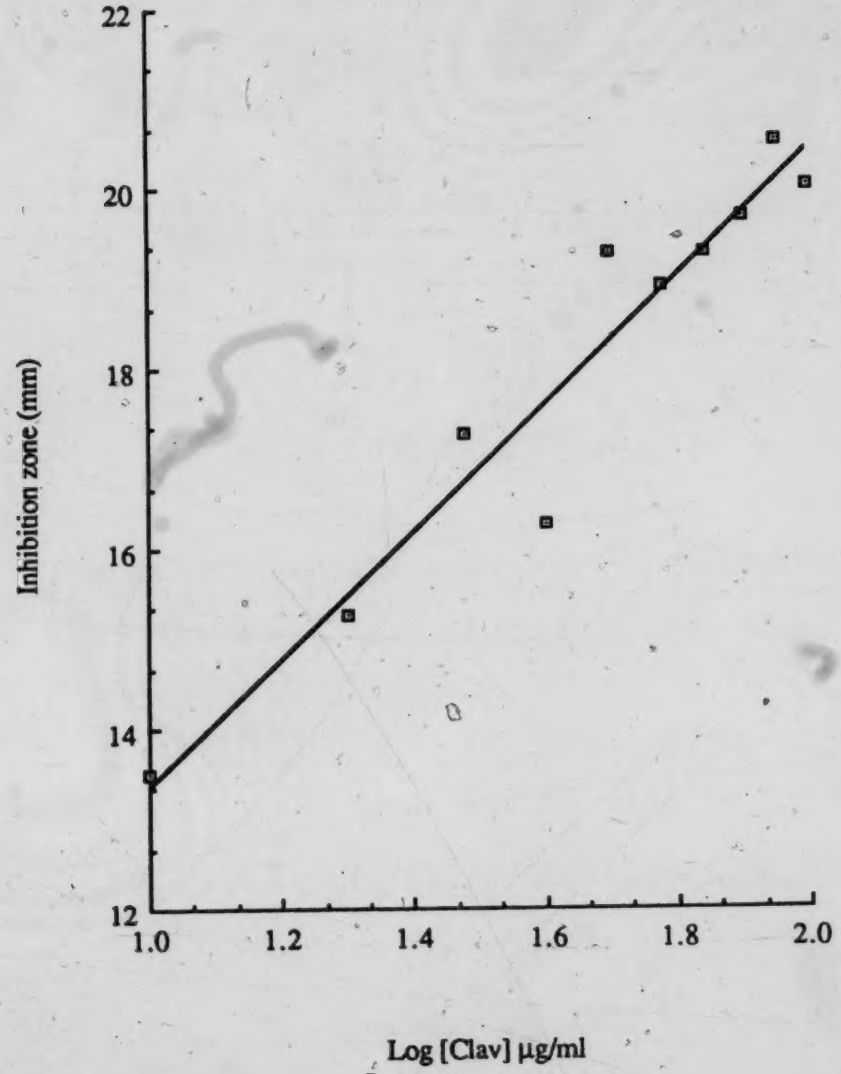
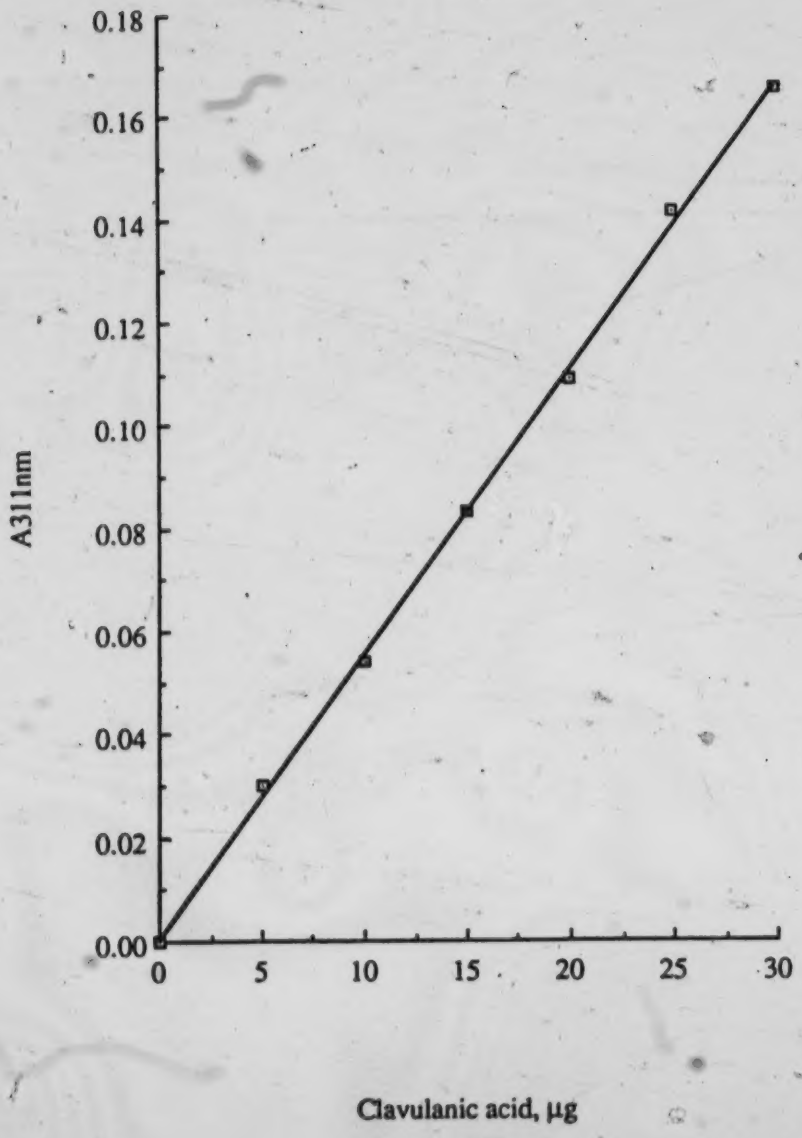

22
20
18
16
14
12
Inhibition zone (mm)
1.0
1.2
1.4
1.6
1.8
2.0
Log [Clav] µg/ml

Figure 2. Standard curve of clavulanic acid as determined by the chemical assay method.


0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
A311nm
0
5
10
15
20
25
30
Clavulanic acid, µg

H_2O or uninoculated production medium. The imidazole-derivatized clavulanic acid can be detected as a single peak in a ultra violet (uv) scan with an absorbance maximum at 311 nm (Figure 3).

B.2. Chemical Assay of Clavulanic Acid in Culture Filtrates.

The usefulness of the chemical assay method for analysis of clavulanic acid in culture filtrates was investigated by reacting a sample of culture filtrate known to contain about 25 μg clavulanic acid/ml as determined by the bioassay. The chemical assay indicated that the culture filtrate contained no clavulanic acid (data not shown). The reason for the discrepancy between these two values was investigated by comparing the UV scans of the derivatized clavulanic acid standards and controls with those of the actual culture filtrates. As shown in figure 4, the imidazole reagent, the uninoculated production medium and the uninoculated production medium reacted with imidazole reagent (curve 1, 2 and 3), had little UV absorbance in the wavelength range of 240 nm to 400nm. In contrast the UV scans of the sample of culture filtrate known to have clavulanic acid from the bioassay, had very high UV absorbance. However, even without imidazole derivatization, the culture filtrate had an absorbance of 0.293 at 311nm (curve 4 figure 4). When this filtrate was reacted with imidazole, there was no absorbance maximum at 311nm but rather, a plateau with an absorbance at 311nm of 0.444 (curve 5). These results indicate that the culture filtrate itself could interfere with the assay.

Further scans were done to determine the nature of the interference (figure 5). Again, the first three controls; imidazole reagent, uninoculated production medium and

Figure 3. Ultraviolet scan of imidazole treated clavulanic acid standards.

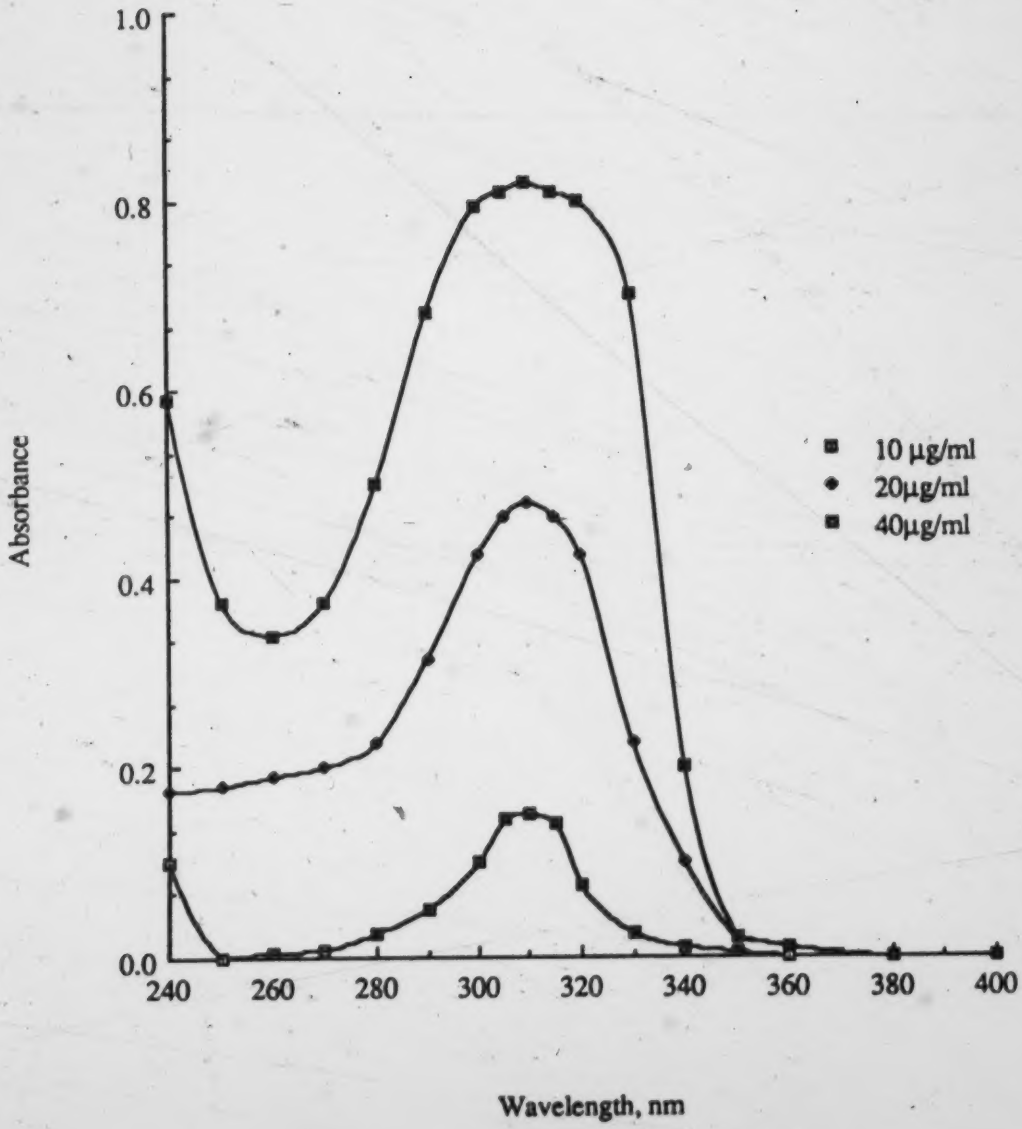

1.0
0.8
0.6
0.4
0.2
0.0
Absorbance
240
260
280
300
320
340
360
380
400
Wavelength, nm
10 µg/ml
20µg/ml
40µg/ml

Figure 4. Ultraviolet scans of untreated and imidazole treated culture filtrates. 1) Imidazole reagent and H2O. 2) Uninoculated production medium. 3) Uninoculated production medium and imidazole reagent. 4) Culture filtrate. 5) Culture filtrate and imidazole reagent.

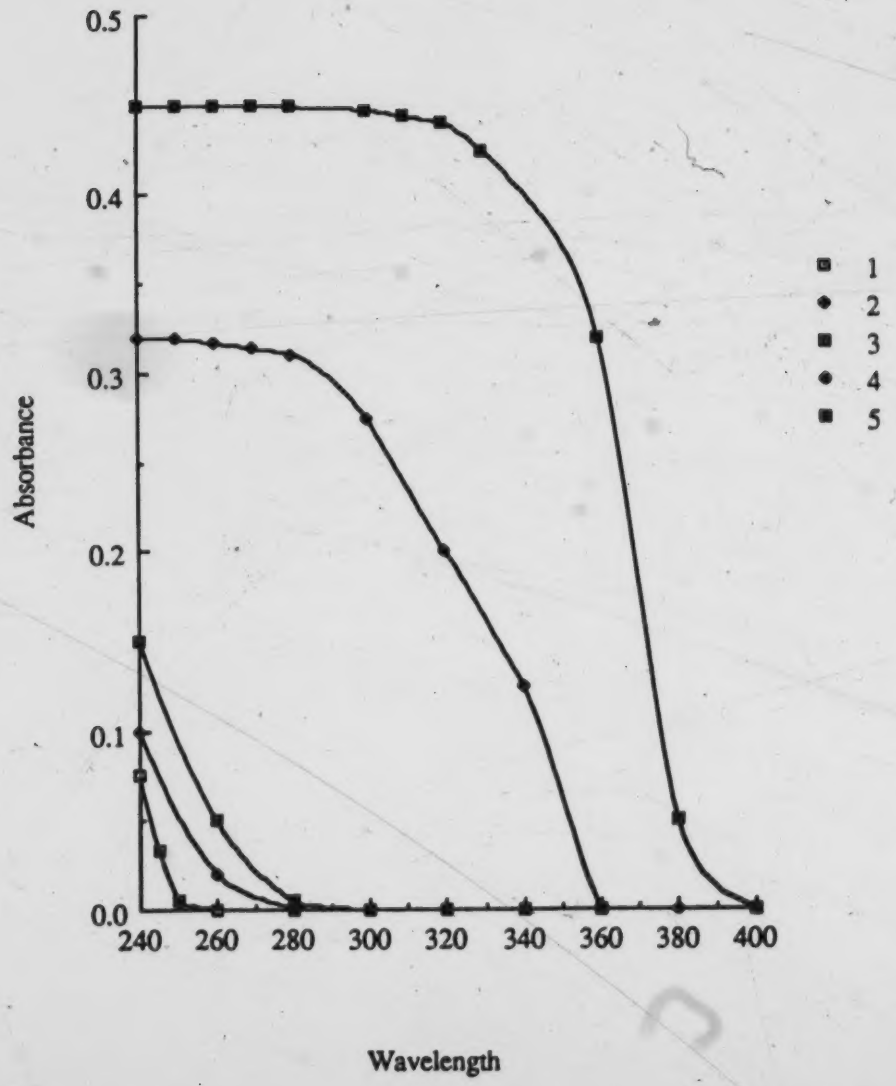
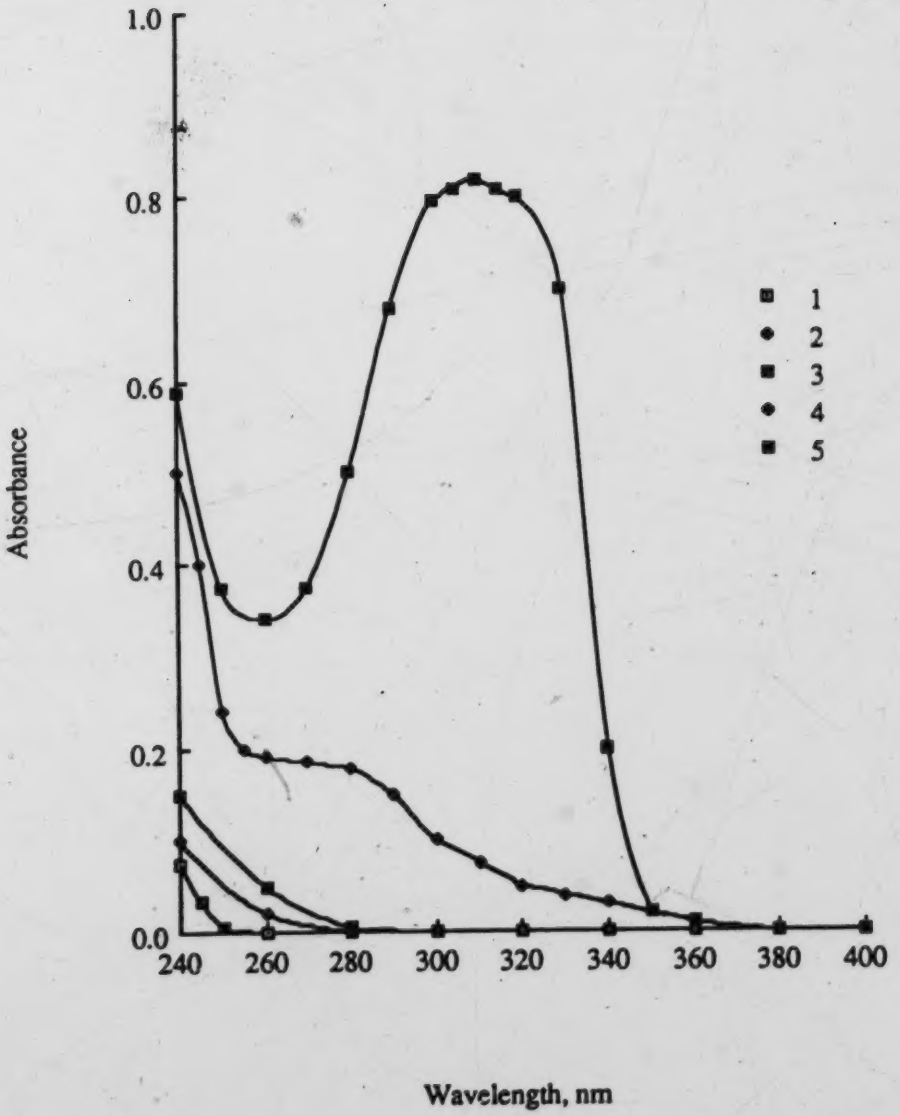

0.5
0.4
0.3
0.2
0.1
0.0
Absorbance
240
260
280
300
320
340
360
380
400
Wavelength
1
2
3
4
5

Figure 5. Ultraviolet scans of untreated and imidazole treated clavulanic acid standards in uninoculated production medium. 1) imidazole reagent. 2) Uninoculated medium. 3) Uninoculated medium with imidazole reagent. 4) Uninoculated production medium with clavulanic acid at 40 μg/ml final concentration. 5) Uninoculated production medium with clavulanic acid at 40 μg/ml final concentration and imidazole reagent.


1.0
0.8
0.6
0.4
0.2
0.0
Absorbance
240
260
280
300
320
340
360
380
400
Wavelength, nm
1
2
3
4
5

uninoculated medium reacted with imidazole (curves 1, 2 and 3), showed a low UV absorbance. Uninoculated production medium supplemented with clavulanic acid standard to a final concentration of 40 μg/ml still had a low uv background (curve 4). When clavulanic acid supplemented production medium was reacted with imidazole (curve 5), a single peak was obtained at 311nm. This suggested that the interfering material was not present in uninoculated production medium.

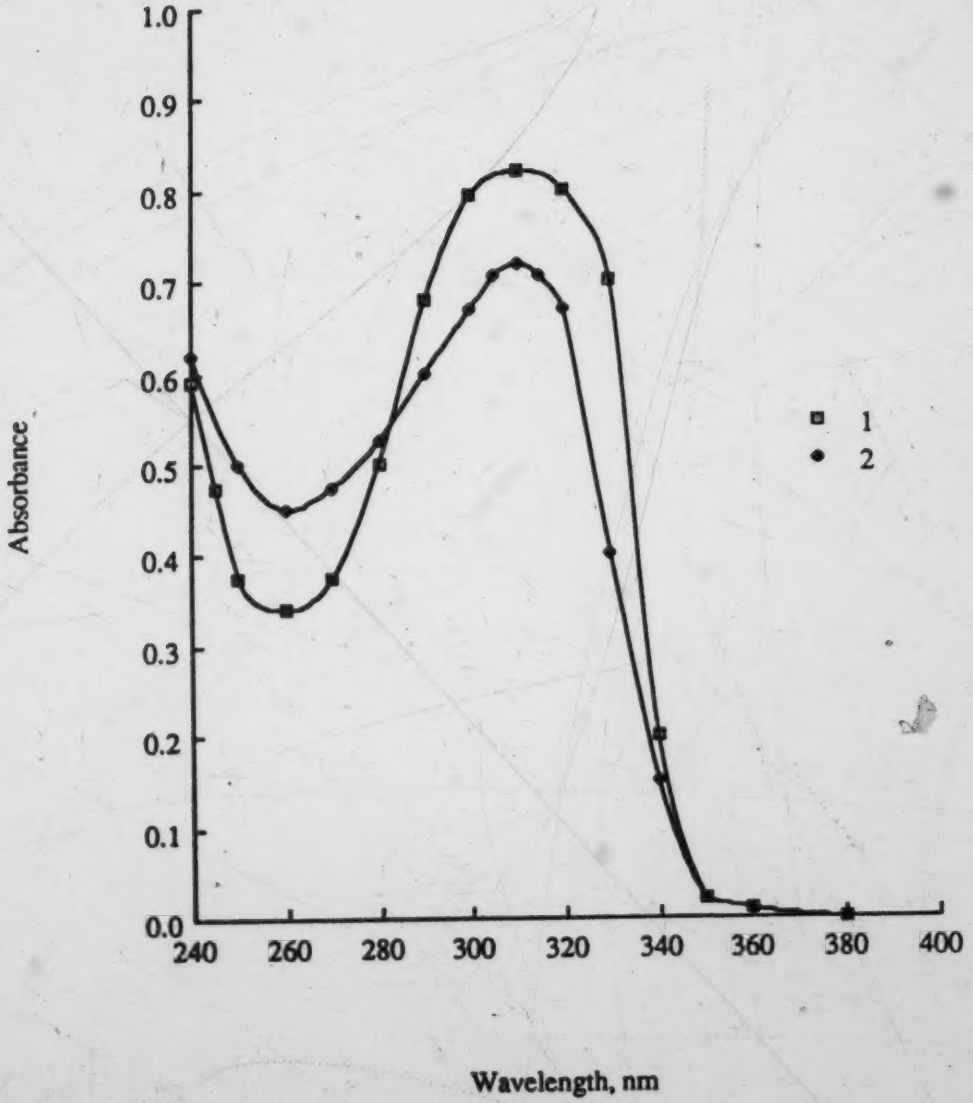
When the culture filtrate was supplemented with clavulanic acid standard to give an additional 40 μg/ml of clavulanic acid, and then was reacted with imidazole, there was a peak at 311nm but the absorbance was lower than expected (curve 2, figure 6). Since there was clavulanic acid initially present, the supplementation of the culture filtrate should give a higher absorbance after reacting with imidazole than the clavulanic acid standard in uninoculated production medium but it did not.

Further evidence that the culture filtrates could interfere with the assay was noted during preliminary nutritional studies. When the carbon and nitrogen sources of the standard production medium were changed, some of the untreated culture filtrate controls gave higher absorbance readings at 311nm than the corresponding imidazole treated culture filtrates (data not shown). Because of these problems, the chemical assay was determined to be unsuitable for analyzing clauvulanic acid production in this study.

C. High Performance Liquid Chromatography (HPLC) analysis of clavulanic acid.

Several HPLC systems were investigated for quantitating clavulanic acid. Initial analyses utilized a mobile phase of 50 mM KH_2PO_4, pH 3.2 or 4.0; and a reversed phase column as described in Materials and Methods. Clavulanic acid standards in

Figure 6. Ultraviolet scans of culture filtrates supplemented with clavulanic acid. 1) Uninoculated production medium with clavulanic acid at 40 µg/ml final concentration and imidazole reagent. 2) Culture filtrate supplemented with clavulanic acid, 40 µg/ml additional concentration and imidazole reagent.


1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Absorbance
240
260
280
300
320
340
360
380
400
1
2
Wavelength, nm

solutions of distilled H_2O or production medium and culture filtrates were poorly resolved and no single peak correlating to clavulanic acid was detectable (data not shown). Clavulanic acid was poorly retained under these chromatographic conditions and the retention time of clavulanic acid was close to the non retained fraction. Clavulanic acid in the culture filtrate could not be resolved from the other medium components.

Modification of the mobile phase by the incorporation of the ion pairing agent Pic A improved the retention of clavulanic acid. This change in the mobile phase also increased the resolution of the peaks in the culture filtrates but clavulanic acid still could not be resolved from the other medium components (figure 7). The Pic A reagent appeared to be increasing the retention of the contaminating peaks in the same way it had increased the retention time of clavulanic acid. When the culture filtrate chromatograms (figure 7b) were compared with the chromatograms of 0.1 M MOPS buffer, pH 6.8, it was determined that the main interfering material was the MOPS buffer (figure 7c). Since MOPS was a necessary buffering component of the production medium, a new analysis system was investigated.

Interference by MOPS was relieved by carrying out a pre-column derivatization of samples with imidazole reagent. Reacting filtrates with the imidazole reagent also improved the retention and separation of clavulanic acid from the medium components (Figure 8a). The retention time of the derivatized peak ranged from 6.3 -7 minutes with a mobile phase of 6% methanol; 94 % 0.1M KH_2PO_4 (adjusted to pH 3.2 with H_3PO_4). Values obtained were very reproducible. Derivatized clavulanic acid standards

Figure 7a. HPLC analysis of clavulanic acid standard using a mobile phase containing PIC A reagent. Five microlitres of 5 µg/ml clavulanic acid standard was analyzed. Mobile phase, 10% methanol + 3% PIC A reagent; 90% 50 mM KH_2PO_4, pH 4.0. Stationary phase, Brownlee C-18 column. λ=214nm. Flow rate= 0.5ml/min.

Figure 7b. HPLC analysis of a culture filtrate using a mobile phase containing PIC A reagent. Five microlitres of a culture filtrate estimated to contain about 40 µg/ml clavulanic acid (by bioassay) was analyzed. Mobile phase, 10% methanol + 3% PIC A reagent ; 90% 50 mM KH_2PO_4 pH 4.0. Stationary phase, Brownlee C-18 column. λ=214nm. Flow rate= 0.5ml/min.

Figure 7c. HPLC analysis of MOPS buffer. Five microlitre of 0.1M MOPS buffer, pH 6.8 was analyzed. Mobile phase, 10% methanol + 3% PIC A reagent; 90% 50 mM KH_2PO_4, pH 4.0. Stationary phase, Brownlee C-18 column. λ=214nm. Flow rate= 0.5ml/min.

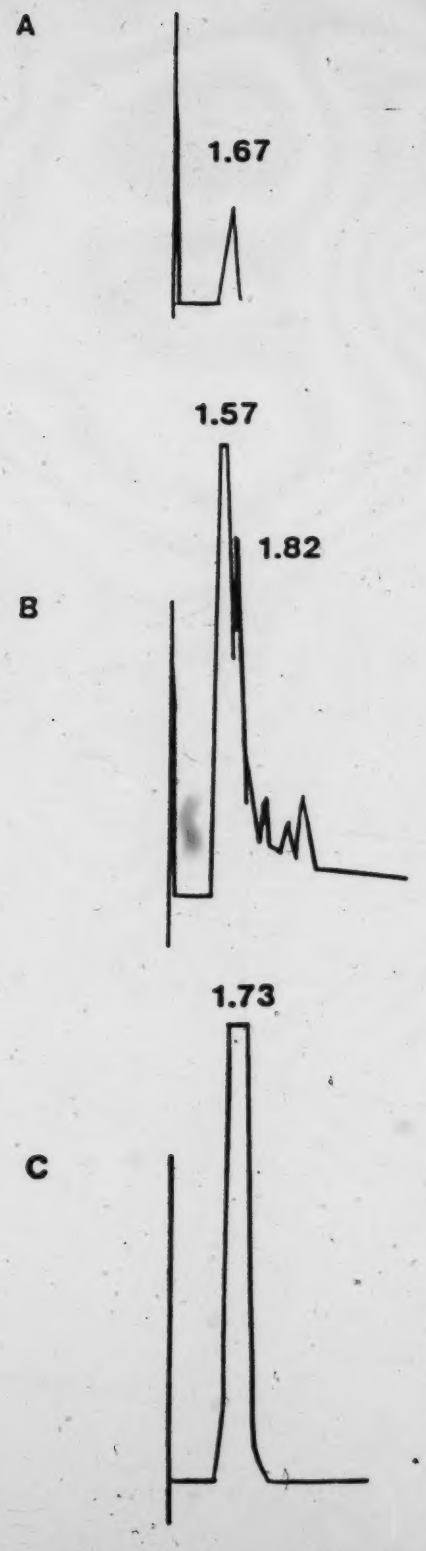
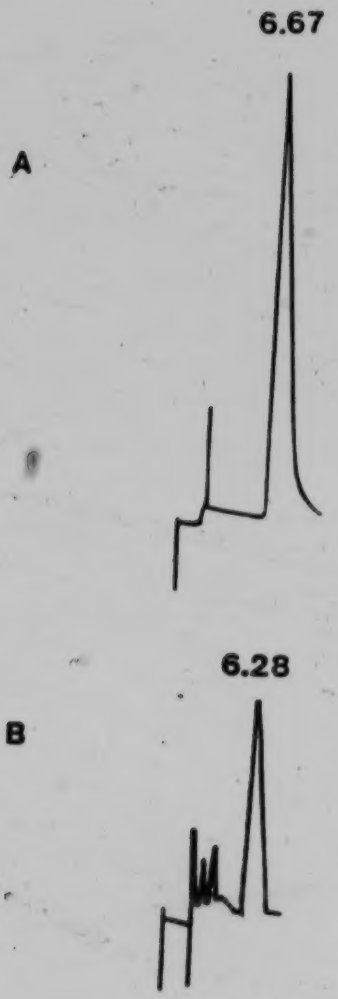

A
1.67
1.57
1.82
B
1.73
C

Figure 8a. HPLC analysis of derivatized clavulanic acid standard. Ten microlitre of a 50 µg/ml clavulanic acid standard was analyzed. Mobile phase, 6% methanol; 94% 100mM KH_2PO_4, pH 3.2. Stationary phase, µ Bonda-pak C-18 column. λ=311nm. Flow rate= 2.0 ml/min.

Figure 8b. HPLC analysis of derivatized culture filtrate. Ten microlitreas of culture filtrate, derivatized with imidazole, was analyzed. Mobile phase, 6% methanol; 94% 100mM KH_2PO_4, pH 3.2. Stationary phase, µ Bonda-pak C-18 column. λ=311nm. Flow rate= 2.0 ml/min.


6.67
A
6.28
B

gave a linear response when the peak areas were plotted against the amount of derivatized clavulanic acid injected. Analysis of the filtrate from a culture grown in standard production medium resulted in chromatograms with good resolution and a sharply defined peak (Figure 8b). Although resolution of clavulanic acid from other components of the culture was good when cultures were grown on the standard production medium described in Materials and Methods, interfering peaks were again encountered when alternative carbon and nitrogen sources were tested (data not shown).

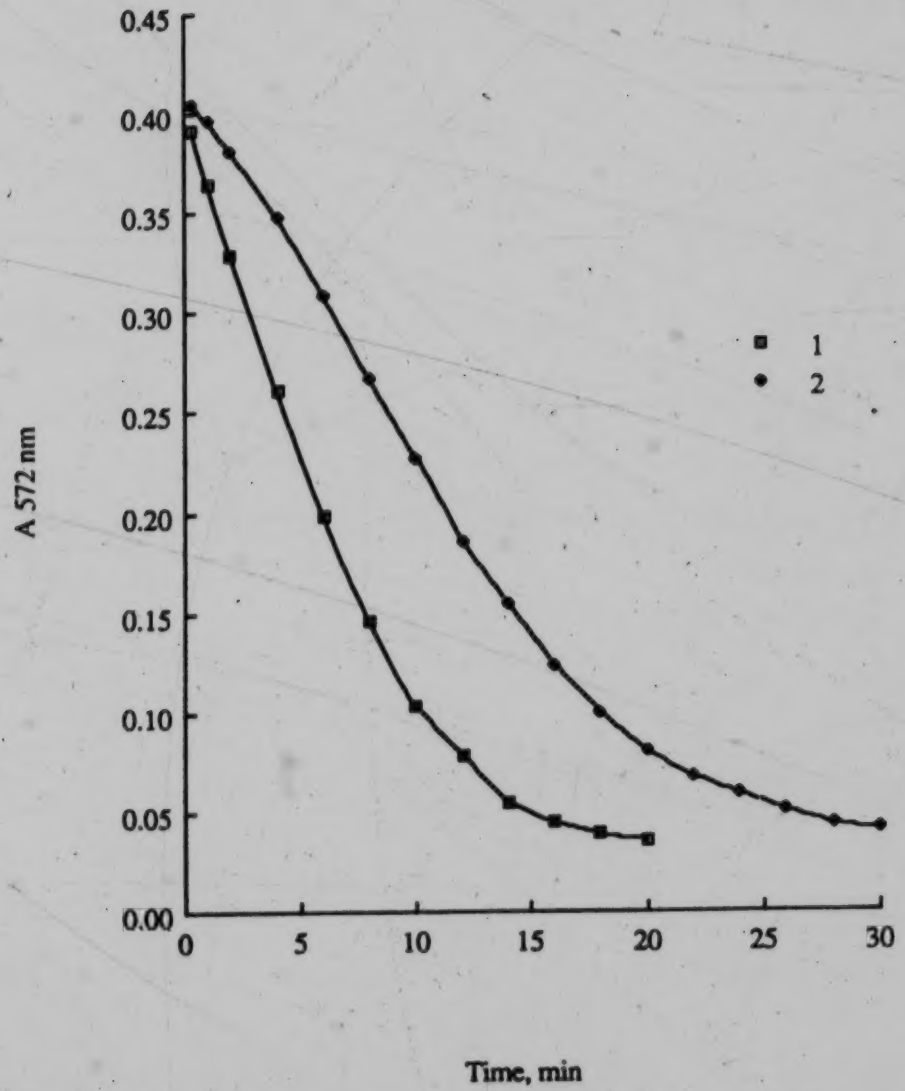
D. Enzyme assays.

Since the biological, chemical and HPLC assays for clavulanic acid analysis all had disadvantages when used for the analysis of culture filtrates, an enzyme based ß-lactamase inhibition assay was investigated. Two different ß-lactam substrates were assessed, but in each case the assay was based on the ability of clavulanic acid to cause the inhibition of the ß-lactamase enzyme. The inhibition was monitored as a decrease in the rate of degradation of the ß-lactam substrate.

The first system utilized a coloured ß-lactam substrate, PADAC, which changes upon hydrolysis by ß-lactamase. Degradation of the PADAC substrate can be monitored by following the decrease in absorbance at 572nm. Without any clavulanic acid added to the ß-lactamase, the rate of degradation of the PADAC substrate was fast (curve 1, figure 9a). Total degradation of the substrate was achieved by 12 minutes. The addition of clavulanic acid to a final concentration of 3.88 µg/ml decreased the rate of degradation and extended the time required for total degradation to 30 minutes (curve 2 figure 9a).

The specificity of the PADAC assay was examined using cephamycin C and

Figure 9a. Degradation of PADAC by ß-lactamase. One hundred thousand units of ß-lactamase was combined with PADAC at a final concentration 3.88 µg/ml (curve 1). The inhibition of ß-lactamase was determined by combining clavulanic acid at a final concentration of 2.5 µg/ml with the enzyme before adding it to the PADAC substrate (curve 2).


0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
A 572 nm
0
5
10
15
20
25
30
Time, min
1
2

clavulanic acid as inhibitors of the ß-lactamase enzyme. The reason for the inclusion of cephamycin C (5.0 µg/ml), a cephalosporin, was that since cephamycin C is a product of the fermentation as well as clavulanic acid, it could possibly interfere with the assay by virtue of being similar in structure to the PADAC substrate. The ß-lactamase enzyme was incubated with PADAC in the presence and absence of clavulanic acid and cephamycin C. If cephamycin C does not interfere with the reaction of the enzyme on the PADAC substrate, then the rate of degradation should be identical to the enzyme control (curve 1, figure 9b). The addition of cephamycin C did have an inhibitory effect on the enzyme but not to the same extent as clavulanic acid. Cephamycin C extended the time required for total degradation of the substrate to 20 minutes (curve 3, figure 9b).

To avoid this interference caused by ß-lactams compounds other than clavulanic acid which are present in the filtrate, the use of a mutant which does not produce penicillins and cephalosporins was examined. The mutant was still able to produce clavulanic acid. Bioassays of the *S. clavuligerus* NTG #12 culture filtrate indicated production of clavulanic acid when grown in a complex medium but the use of this mutant was discontinued because it was unable to grow in the production medium used for nutritional studies (data not shown).

The second enzyme assay that was used to quantitate clavulanic acid was the method described by Reading and Hepburn, and Gutman *et al.*, (Reading and Hepburn, 1979, and Gutman *et al.*, 1985b). The substrate is penicillin G, and the absorbance of penicillin G at 240nm decreases upon hydrolysis by ß-lactamase. The addition of clavulanic acid inhibits the ß-lactamase enzyme therefore decreasing the rate of degradation of the penicillin G substrate (figure 10). Quantitation can be achieved by

Figure 9b. Specificity of the PADAC assay. Curves 1 and 2 are the same as Figure 9a. The inhibitory effect of cephamycin C on ß-lactamase activity was determined by combining cephamycin C at a final concentration of 1.25 µg/ml with the enzyme before adding it to the PADAC substrate (curve 3).

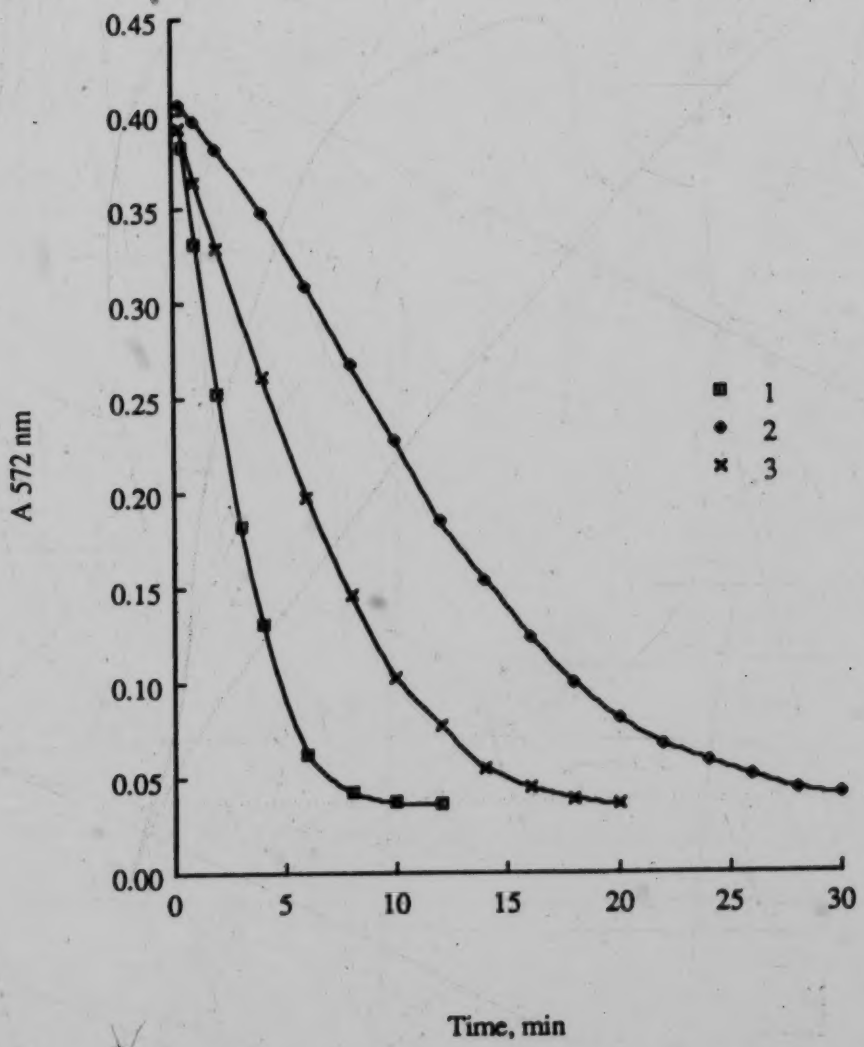
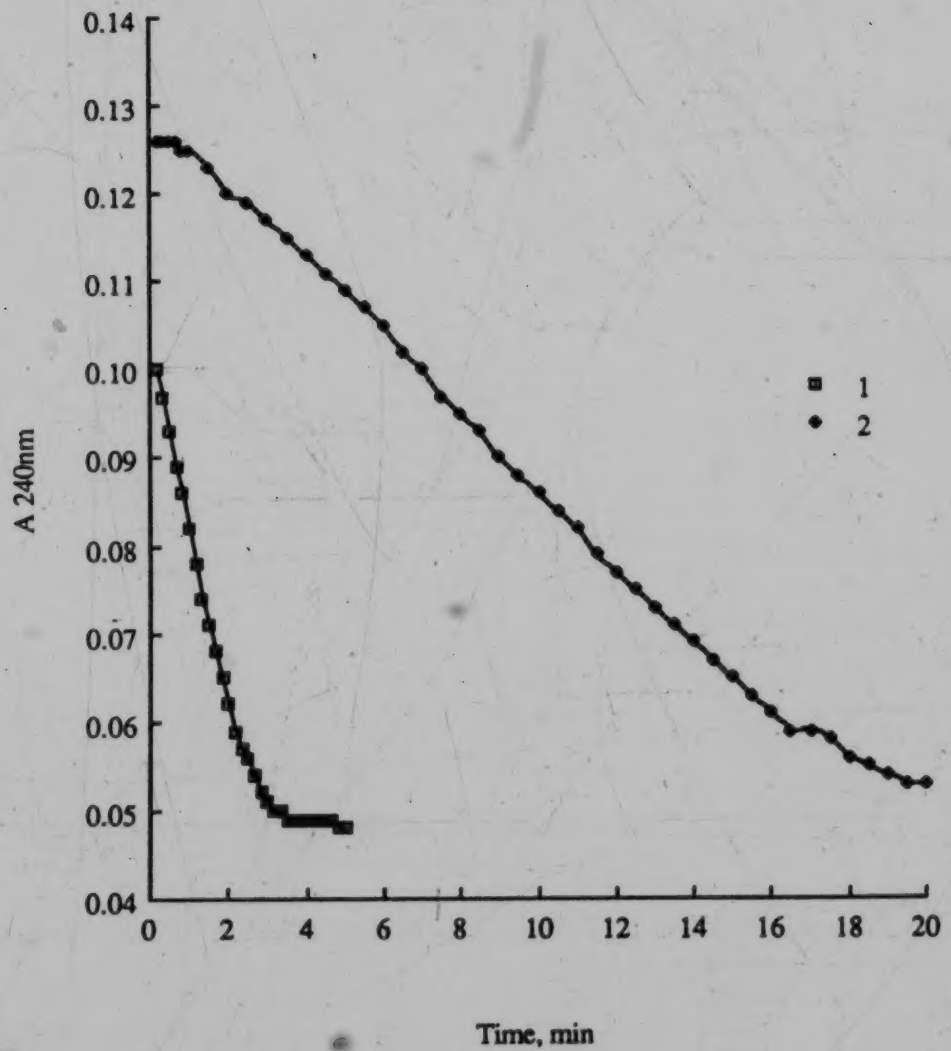

0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
A 572 nm
0
5
10
15
20
25
30
Time, min
1
2
3

Figure 10. Degradation of penicillin G by ß-lactamase. One hundred thousand units of penicillinase was combined with penicillin G at a final concentration 25 µg/ml (curve 1). The inhibition of ß-lactamase was determined by combining clavulanic acid at a final concentration of 1.25 µg/ml with the enzyme before adding it to the penicillin G substrate (curve 2).


0.14
0.13
0.12
0.11
0.10
0.09
0.08
0.07
0.06
0.05
0.04
A 240nm
0
2
4
6
8
10
12
14
16
18
20
Time, min
1
2

plotting the percentage of rate inhibition against the amount of clavulanic acid. The resulting curve is non linear, as previously described by Gutman *et al.* (1985b) (figure 11a). Interpolation from such a curve was found to be difficult. To minimize the problem, a plot of the percentage of rate inhibition against the third root of the amount of clavulanic acid was found to result in a standard curve that was linear (figure 11b), making interpolations more precise.

Unlike the PADAC assay, cephamycin C did not interfere with this reaction. There was no decrease in the rate of degradation when cephamycin C was substituted for clavulanic acid (figure 12, curve 3). This was probably due to the higher concentration of penicillin G used in the assay and that the enzyme has a higher specific activity as a penicillinase than a cephalosporinase therefore showing greater specificity to penicillin G. The culture filtrate itself did have some interference. Filtrates that tested negative for clavulanic acid by the bioassay, inhibited the rate of degradation indicating the presence of clavulanic acid. The interference problems were alleviated by the addition of equal volumes of methanol to the culture filtrates. The addition of methanol eliminated the false positive results. Standard solutions of clavulanic acid were not affected with the methanol addition. The ß-lactamase was not affected by the methanol. The filtrates alone with the penicillin G substrate showed no ß-lactamase enzyme activity. Unless otherwise indicated, all subsequent studies were done with this assay.

Figure 11a. Standard curve of the inhibition of penicillin G by clavulanic acid. Plot is the percentage of rate inhibition against the amount of clavulanic acid assayed. Fifteen thousand units of penicillinase (Difco type I) was combined with 0 - 10 μg of clavulanic acid in a final volume of 0.5 ml of 0.1M sodium phosphate buffer, pH 6.8. After the preincubation, 500 μL of 300 μg/ml penicillin G was added to the mixture and the rate of degradation was followed at 240nm.

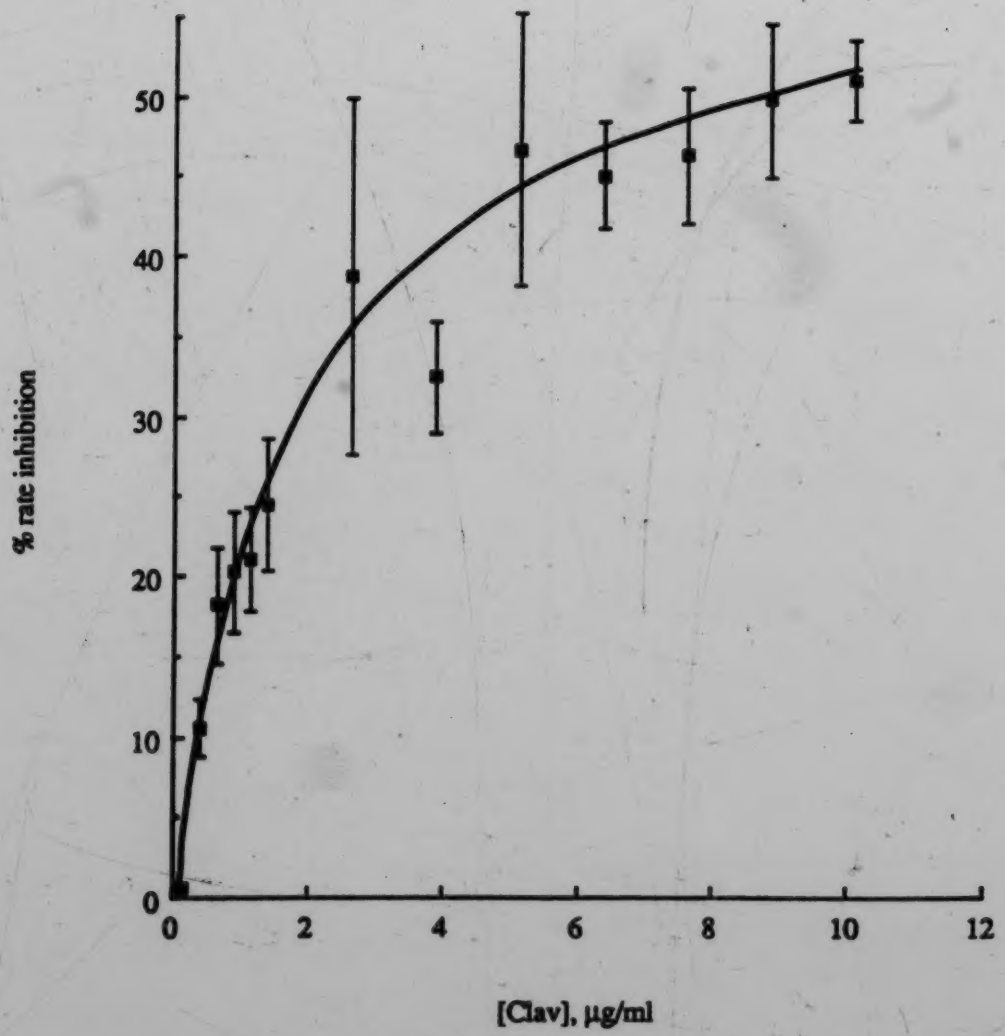

% rate inhibition
0
10
20
30
40
50
0
2
4
6
8
10
12
[Clav], µg/ml

Figure 11b. Standard curve of the inhibition of penicillin G by clavulanic acid. Plot is the percentage of rate inhibition against the third root of the amount of clavulanic acid assayed.

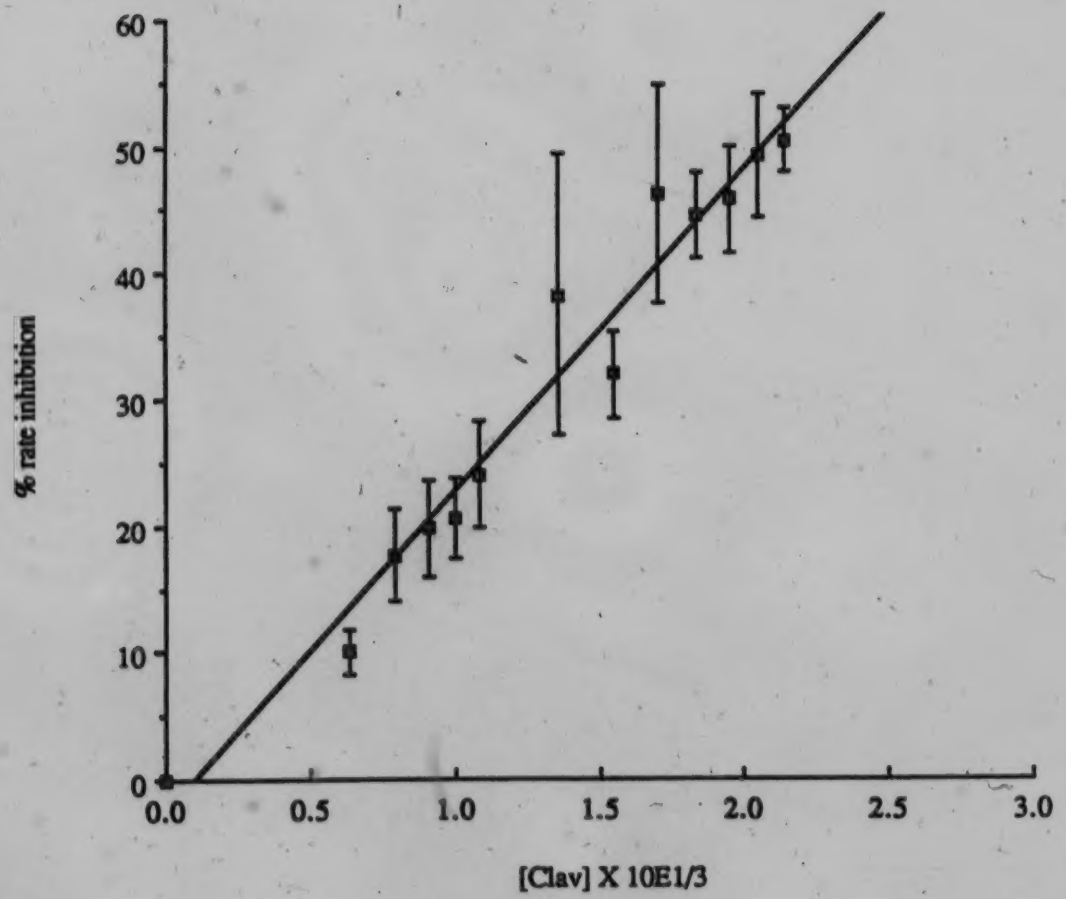
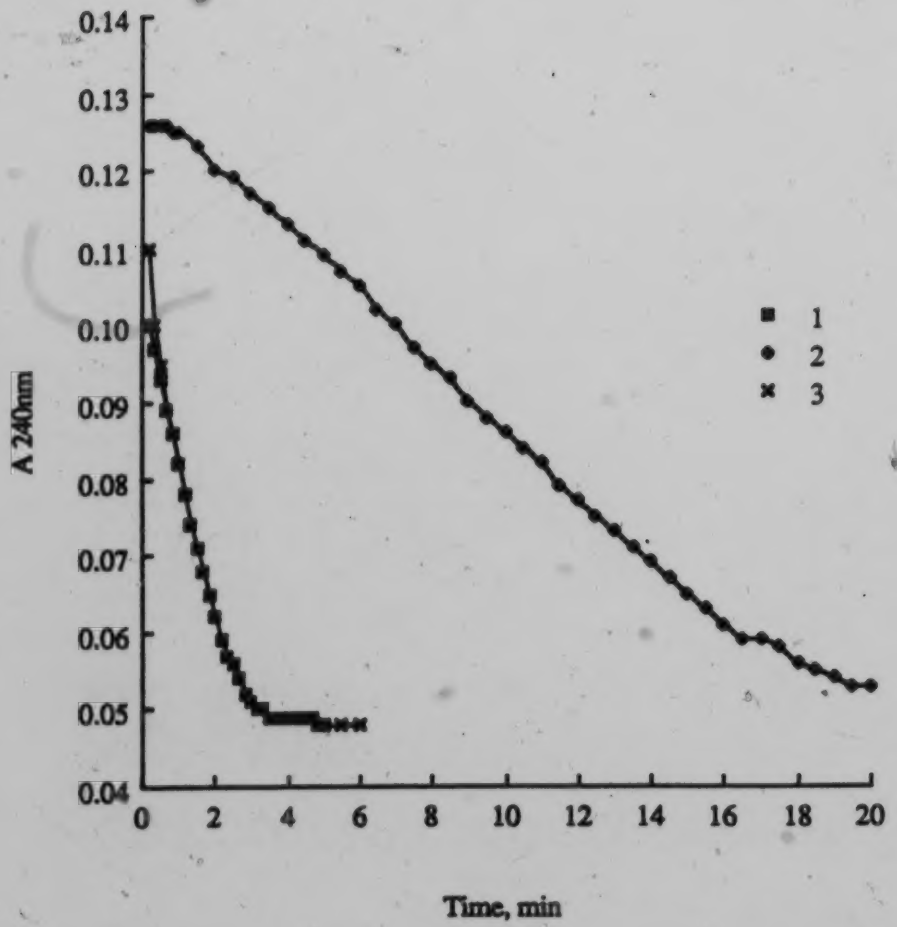

% rate inhibition
60
50
40
30
20
10
0
0.0
0.5
1.0
1.5
2.0
2.5
3.0
[Clav] X 10E1/3

Figure 12. Specificity of the penicillin G assay. Curves 1 and 2 are the same as Figure 10. The inhibitory effect of cephamycin C on ß-lactamase activity was determined by combining cephamycin C at a final concentration of 1.25 µg/ml with the enzyme before adding it to the penicillin G substrate (curve 3).


0.14
0.13
0.12
0.11
0.10
0.09
0.08
0.07
0.06
0.05
0.04
A 240nm
0
2
4
6
8
10
12
14
16
18
20
Time, min
1
2
3

IV. 2. NUTRITIONAL STUDIES

A. CARBON SOURCES

The effect of different carbon sources on clavulanic acid production was examined by replacing the glycerol in the production medium with a variety of other compounds. Initially, 10 ml amounts of culture filtrates were sampled every 24 h for 168 h. The culture filtrates were assayed for clavulanic acid by the bioassay. The carbon sources most suitable for growth and the production of clavulanic acid of *S. clavuligerus*, were found to be maltose, glycerol, dextrin and starch (table 1). There was no growth or production of clavulanic acid with organic acids such as succinate and lactate or with sugars such as ribose, glucose, galactose and lactose.

To determine which carbon source promoted the best clavulanic acid production, starch, glycerol, maltose and dextrin were investigated more closely (table 2). Succinate and lactate containing cultures were also reexamined because Aharonowitz and Demain had reported that these carbon sources supported good levels of cephalosporin production by *S. clavuligerus*. Culture filtrates were sampled in triplicate at three time periods. The clavulanic acid content of the culture filtrates was determined by the penicillin G enzyme assay. As with the earlier results, succinate and lactate did not support growth and promoted poor production of clavulanic acid. Starch, glycerol, maltose and dextrin were all able to support growth and promote production of clavulanic acid. The levels of total clavulanic acid production were similar for all four of these carbon sources. In the first fermentation run (table 2a), a maximum of 157 μg/ml of clavulanic acid was produced with maltose or starch as the carbon source. Maximum production was acheived with starch and dextrin as the carbon source in the second

Table 1. Carbon source utilization of *Streptomyces clavuligerus*.

Carbon Source*	Growth	Clavulanic acid Production
Ribose	±	-
Succinate	±	-
Maltose	+	+
Glucose	-	-
Lactose	-	-
Galactose	-	-
Lactate	-	-
Starch	+	+
Glycerol	+	+
Dextrin	+	+

(±) = Scant growth
*Carbon sources were added at 0.5% w/v

Table 2a. Clavulanic acid Production with Various Carbon Sources.

Carbon Source*	time(h)	Dry Cell Weight (DCW) mg/ml	std dev	[Clavulanic acid] µg/ml	std dev	Specific Production µg/mg	std dev
Succinate	24	0.660	0.185	0.000	0.000	0.000	0.000
	50	1.06	0.140	0.409	0.012	0.386	0.137
	96	0.720	0.069	0.052	0.007	0.072	0.000
Lactate	24	0.903	0.090	0.156	0.004	0.173	0.103
	50	0.757	0.099	0.506	0.047	0.669	0.162
	96	0.907	0.099	2.89	0.111	3.19	0.116
Maltose	24	1.36	0.014	0.077	0.006	0.057	0.000
	50	2.38	0.045	13.5	0.189	5.68	0.024
	96	4.84	1.72	157	4.76	32.4	0.391
Starch	24	1.48	0.200	1.58	0.052	1.07	0.140
	50	3.59	0.245	82.0	4.05	22.9	0.084
	96	4.27	0.082	157	2.20	36.8	0.024
Glycerol	24	2.58	0.186	2.64	0.138	1.02	0.089
	50	4.38	0.565	140	10.4	32.0	0.150
	96	4.26	0.119	70.3	1.74	16.5	0.037

*Carbon sources were added at 1% (w/v).
Dry Cell Weight (DCW) were done in triplicate for each sample time.
Clavulanic acid determinations were done nine times per sample time.
Dry Cell Weight (DCW) at time zero, 0.631mg/ml.

fermentation run (table 2b). In the second fermentation run (table 2b), overall production levels were similar. In both fermentations, production of clavulanic acid was slightly lower with glycerol as the carbon source (129-140 μg/ml), and maximum production occurred at 48-96 hours into the fermentation. Despite the slightly lower production levels, glycerol was still chosen as the preferred carbon source to work with in further studies because of several factors: 1) previous studies have used glycerol as the carbon source in studying both cephasporin C production and clavulanic acid production (Aharonowitz and Demain, 1977 and Romero *et al.*, 1984); and 2) dextrin and starch are less defined as carbon sources.

B. GLYCEROL

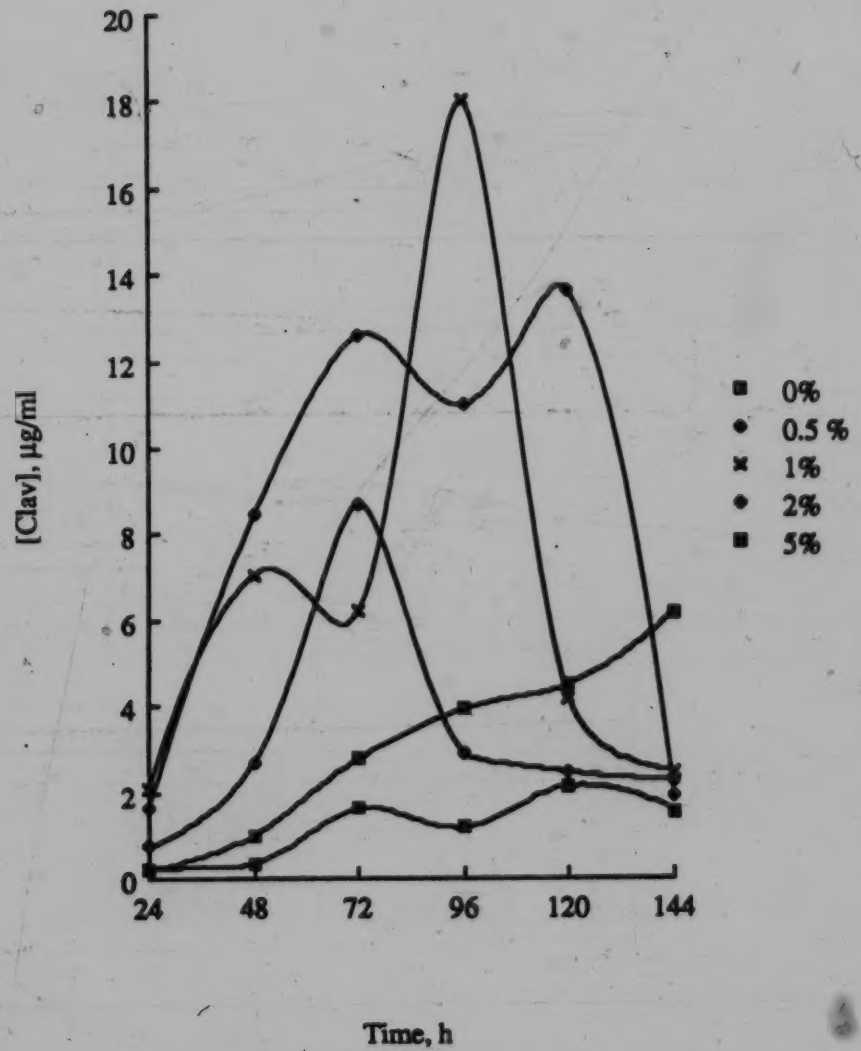
To determine if carbon catabolite regulation plays a role in clavulanic acid production, different amounts of glycerol concentrations were added to the production medium. The glycerol contents tested were 0, 0.5, 1, 2 and 5% (w/v). Initially, the culture filtrates were sampled every 24 h during a one week period. The clavulanic acid content was determined by the pre-column derivatization HPLC assay. Although the HPLC method of analysis was determined to be not reliable, the pattern of clavulanic acid production was similar to subsequent studies. The results revealed that in the absence of glycerol, there was low levels of clavulanic acid being produced (figure 13). The first detectable levels were obtained with 0.5 % glycerol as the carbon source. At glycerol concentrations greater than 1%, the levels of clavulanic acid production decreased. The maximum production of clavulanic (18 μg/ml) was obtained with 1% glycerol and an incubation time of 96 h. Although the production levels varied, this pattern has been repeated.

Table 2b. Repeat of Clavulanic acid Production with Various Carbon Sources.

Carbon Source*	time(h)	Dry Cell Weight (DCW) mg/ml	std dev	[Clavulanic acid] µg/ml	std dev	Specific Production µg/mg	std dev
Succinate	24	1.24	0.060	2.21	0.126	1.79	0.075
	48	2.10	0.062	0.288	0.046	0.137	0.163
	96	2.90	0.202	0.288	0.015	0.099	0.088
Dextrin	24	1.55	0.101	8.41	0.565	5.44	0.094
	48	4.02	0.070	26.5	1.44	6.59	0.057
	96	4.22	0.026	174	11.7	41.2	0.068
Maltose	24	1.40	0.488	0.366	0.021	0.261	0.378
	48	1.89	0.100	39.9	2.32	21.1	0.079
	96	4.43	0.012	157	10.3	35.5	0.066
Starch	24	1.79	0.095	9.17	0.567	5.12	0.082
	48	4.04	0.135	161	14.0	39.9	0.093
	96	3.47	0.007	152	9.66	43.9	0.064
Glycerol	24	2.19	0.050	2.21	0.122	1.01	0.060
	48	4.82	0.265	129	7.53	26.7	0.080
	96	3.57	0.069	89.9	5.13	25.2	0.060

*Carbon sources were added at 1% (w/v).
Dry Cell Weight (DCW) were done in triplicate for each sample time.
Clavulanic acid determinations were done nine times per sample time.

Figure 13. The production of clavulanic acid in response to glycerol concentrations ranging from 0-5%w/v as the carbon source. The nitrogen source was 0.2 g/100ml asparagine. The phosphate source was 25mM KH_2PO_4. The production medium constituents is as listed in the material and methods. The method of analysis was the pre-column HPLC derivatization of clavulanic acid.


20
18
16
14
12
10
8
6
4
2
0
[Clav], µg/ml
24
48
72
96
120
144
Time, h
0%
0.5 %
1%
2%
5%

Although there was a good indication that the glycerol concentration has a effect on the production of clavulanic acid, the low level of clavulanic acid production and the variability between samples were a concern. To solve the variation problem, culture uniformity was optimized by using a two stage inoculum preparation. In the first stage, spore stocks of *S. clavuligerus* were transferred to phytone seed (PS) medium for germination. Maximum vegetative growth occurred after about 48 h incubation at 28°C at 250 rpm. The germinated cells were harvested by filtration through a pre-wetted Whatman #2 filter, washed with sterile distilled H_2O, scraped off and resuspended in distilled H_2O to the original culture volume.

In the second stage, the production medium was inoculated by adding the washed inoculum to double strength production medium to a final concentration of 4% (v/v). The mixture was dispensed in 50ml aliquots into sterile 500ml erlenmeyer flasks containing 50ml of distilled H_2O. When individual components of the production medium were to be varied, double strength production medium was prepared lacking the component to be examined. The inoculated double strength production medium was then dispensed as described as above into flasks containing 50ml of appropiate solutions of the growth medium component to be examined. Triplicate cultures for each growth medium composition were incubated on a New Brunswick platform shaker at 250 rpm at 28°C. Initially, the culture filtrates were pooled and assayed in triplicate, but later, each culture filtrate was analysed in triplicate individually. Even with these precautions, it was still difficult to get consistent levels of clavulanic acid production but the pattern of maximum production appears to be consistent.

When the effect of glycerol on clavulanic acid production was repeated using this standardized inoculum preparation technique and using the penicillin G enzyme assay, the production levels varied but the pattern was similar in each fermentation. Three separate fermentation runs are shown in order to indicate the degree of variability seen between runs (tables 3a-3c). The maximum amount of clavulanic acid produced ranged from 84.7 μg/ml to 169 μg/ml for 1% (w/v) glycerol, which was the best concentration overall. The third fermentation (table 3c), showed 0.5% glycerol to give better production (193 μg/ml), but this appeared to be an unusual result since all other fermentation runs showed 1% glycerol to be the optimum. This confirmed the earlier HPLC results that at higher concentration of glycerol, clavulanic acid production was reduced (figure 14).

Including the data presented in table 3, five separate fermentation runs were carried out to determine the maximum levels of clavulanic acid produced and incubation times required to reach these levels for cultures growing on 1% glycerol as the carbon source (table 4). Under these cultivation conditions, maximum production of clavulanic acid generally occurred at 48 h. The maximum amount of clavulanic acid produced ranged from 84.7 μg/ml to 169 μg/ml (table 4). Based on these studies all subsequent experiments used 1% glycerol as the carbon source.

C. NITROGEN Regulation

The effect of different nitrogen sources on clavulanic acid production was investigated by replacing the asparagine in the production medium with equal amounts

Table 3a. Growth And Clavulanic Acid Production With Different Concentrations of Glycerol. Fermentation #1.

[Glycerol] % (w/v)	time(h)	Dry Cell Weight (DCW) mg/ml	std dev	[Clavulanic acid] µg/ml	std dev	Specific Production µg/mg	std dev
0.5	24	1.43	0.035	0.000	0.000	0.000	0.000
	48	2.32	0.171	38.5	2.20	16.6	1.56
	95	1.88	0.035	56.5	6.12	30.1	3.30
1	24	1.64	0.007	6.57	0.53	4.02	0.323
	48	4.73	0.174	84.7	4.28	17.9	1.12
	95	5.10	0.736	58.9	5.72	11.5	2.08
2	24	3.53	0.369	0.000	0.000	0.000	0.000
	48	5.32	0.123	35.8	3.88	6.73	0.746
	95	4.25	0.425	38.9	2.67	9.15	1.11
5	24	2.71	0.192	6.04	1.49	2.23	0.574
	48	5.03	0.325	0.288	0.09	0.057	0.018
	95	1.17	0.027	4.93	1.04	4.21	0.893

Table 3b. Growth And Clavulanic Acid Production With Different Concentrations of Glycerol. Fermentation #2.

[Glycerol] % (w/v)	time(h)	Dry Cell Weight (DCW) mg/ml	std dev	[Clavulanic acid] µg/ml	std dev	Specific Production µg/mg	std dev
0.5	24	1.10	0.115	0.034	0.042	0.031	0.039
	48	2.90	0.519	57.7	5.49	19.9	4.24
	95	2.67	0.125	51.4	4.89	19.3	2.05
1	24	1.57	0.906	21.0	7.57	13.4	9.52
	48	4.19	0.100	112	13.7	26.7	3.33
	95	3.12	0.035	72.4	6.69	23.2	2.16
2	24	2.45	0.166	0.000	0.000	0.000	0.000
	48	4.34	0.370	16.0	0.476	3.69	0.337
	95	4.47	0.877	4.59	1.09	1.03	0.331
5	24	2.68	0.014	0.034	0.136	0.013	0.051
	48	4.50	0.436	0.581	0.124	0.129	0.031
	95	4.52	0.465	10.5	1.29	2.32	0.377

Table 3c. Growth And Clavulanic Acid Production With Different Concentrations of Glycerol. Fermentation #3.

[Glycerol] % (w/v)	time(h)	Dry Cell Weight (DCW) mg/ml	std dev	[Clavulanic acid] µg/ml	std dev	Specific Production µg/mg	std dev
0.5	24	1.21	0.078	34.8	4.21	28.7	0.137
	48	4.56	0.121	193	19.1	42.4	0.102
	95	2.43	0.070	138	15.9	56.9	0.119
1	24	1.30	0.035	36.2	3.46	27.8	0.099
	48	4.13	0.071	169	17.4	41.0	0.104
	95	3.22	0.046	122	12.7	37.9	0.105
2	24	2.44	0.211	18.9	1.74	7.74	0.126
	48	4.23	0.156	125	12.0	29.6	0.103
	95	5.40	0.122	65.5	7.21	12.1	0.112
5	24	3.25	0.044	4.89	0.444	1.50	0.092
	48	4.24	0.763	20.7	3.14	4.88	0.239
	95	6.33	0.337	3.87	0.598	0.611	0.164

Dry Cell Weight (DCW) were done in triplicate for each sample time.
Clavulanic acid determinations were done nine times per sample time.

Figure 14. Effect of glycerol concentrations on growth and clavulanic acid production in *S. clavuligerus*. The nitrogen source was 0.2 g/100ml asparagine. The phosphate source was 25mM KH_2PO_4.

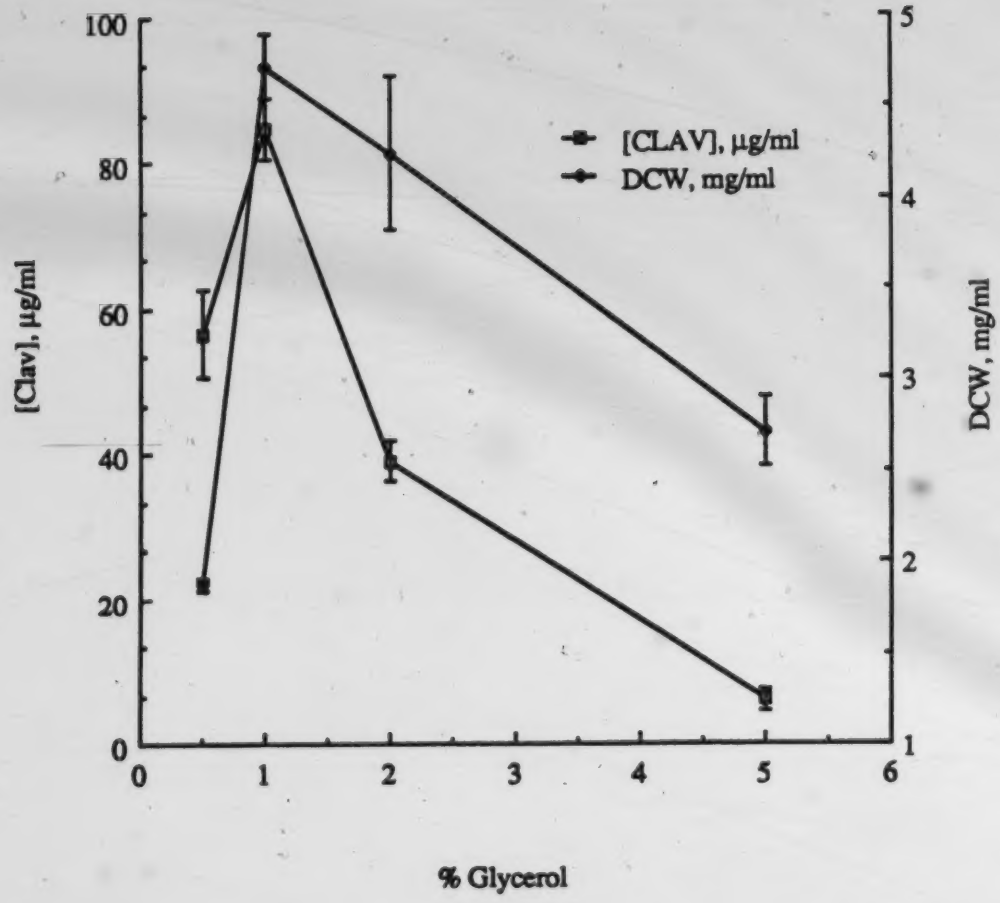

[CLAV], µg/ml
DCW, mg/ml
[Clav], µg/ml
DCW, mg/ml
% Glycerol
0
20
40
60
80
100
1
2
3
4
5
6

Table 4. Comparison of Clavulanic Acid Production with 1% W/V Glycerol.

Fermentation Number	time(h)	Dry Cell Weight (DCW) mg/ml	std dev	[Clavulanic acid] µg/ml	std dev	Specific Production µg/mg	std dev
1	24	1.64	0.007	6.57	0.53	4.02	0.323
	48	4.73	0.174	84.7	4.28	17.9	1.12
	95	5.10	0.736	58.9	5.72	11.5	2.08
2	24	1.57	0.906	21	7.57	13.4	9.52
	48	4.19	0.100	112	13.7	26.7	3.33
	95	3.12	0.035	72.4	6.69	23.2	2.16
3	24	1.30	0.035	36.2	3.46	27.8	0.099
	48	4.13	0.071	169	17.4	41.0	0.104
	95	3.22	0.046	122	12.7	37.9	0.105
4	24	2.58	0.186	2.64	0.138	1.02	0.089
	50	4.38	0.565	140	10.4	32.0	0.150
	96	4.26	0.119	70.3	1.74	16.5	0.037
5	24	2.19	0.050	2.21	0.122	1.01	0.060
	48	4.82	0.265	129	7.53	26.7	0.080
	96	3.57	0.069	89.9	5.13	25.2	0.060

Dry Cell Weight (DCW) were done in triplicate for each sample time.
Clavulanic acid determinations were done nine times per sample time.

of a variety of other nitrogen containing compounds. Although asparagine does have carbon in its structure, making it a possible source of carbon as well as nitrogen, it cannot support growth as a sole source of carbon and energy. When cultures were grown in production medium with only asparagine serving as both the nitrogen and carbon source, there was negligible growth or production of clavulanic acid.

The nitrogen sources tested were urea, aspartic acid, glutamine, $NaNO_3$ and NH_4Cl. The culture filtrates were taken from three time periods (24, 48 and 96h). The initial studies revealed that urea and $NaNO_3$ were unsuitable as nitrogen sources in that they could not support cell growth. The sparse growth seen with urea was lower than reported by other groups (Aharonowitz and Demain, 1979). This may be due to the use of a washed inoculum. Urea and $NaNO_3$ supported relatively low production of clavulanic acid. Aspartic acid, asparagine, and glutamine were found to give good production. Ammonium chloride (NH_4Cl) also supported good production levels (table 5).

A repeat fermentation with samples taken at more frequent intervals indicated that the clavulanic acid production levels were lower with NH_4Cl as the nitrogen source than was seen in the first fermentation run (table 6). The reduction was not as drastic as was determined for cephalosporin production where NH_4Cl reduced production by 75% (Aharonowitz and Demain, 1979). The highest production of clavulanic acid (61.6 µg/ml) was obtained with 0.2 g/100ml aspartic acid as the nitrogen source. Asparagine at the concentration (0.2 g/100ml), produced the next highest amount at 56.6 µg/ml. Glutamine and NH_4Cl produced lower amounts of 36.7and 31.3 µg/ml respectively. The time of

Table 5. Clavulanic Acid Production with Different Nitrogen Sources.

Nitrogen Source	Sample time(h)	Dry Cell Weight (DCW) mg/ml	std dev	[Clavulanic acid] µg/ml	std dev	Specific Production µg/mg	std dev
Asparagine	24	1.37	0.096	0.794	0.122	0.536	0.089
	48	4.60	0.369	22.2	3.61	4.50	0.807
	96	3.96	0.101	26.5	4.51	6.61	1.14
Aspartic Acid	24	1.32	0.084	0.774	0.044	0.569	0.048
	48	3.43	0.090	56.6	1.76	16.7	0.683
	96	3.26	0.052	37.7	1.73	11.5	0.546
Urea	24	0.790	0.044	2.95	0.086	3.60	0.218
	48	0.987	0.127	15.0	2.13	14.0	2.59
	96	0.793	0.117	0.000	0.000	0.000	0.000
Glutamine	24	1.52	0.015	36.7	1.57	24.1	1.06
	48	5.16	0.062	56.6	2.97	11.0	0.592
	96	3.97	0.183	0.774	0.022	0.195	0.011
Sodium Nitrate	24	0.817	0.100	2.01	0.089	2.18	0.256
	48	0.817	0.012	7.41	0.366	8.93	0.458
	96	0.987	0.158	18.3	0.679	21.5	4.08
Ammonium Chloride	24	1.89	0.0551	18.3	0.796	9.84	0.518
	48	3.69	0.341	9.45	0.495	2.34	0.233
	96	4.44	0.236	73.2	2.26	16.2	0.983

Organic nitrogen sources, 2g/L
Inorganic nitrogen sources, 30.4 mM
Dry Cell Weight (DCW) were done in triplicate for each sample time.
Clavulanic acid determinations were done nine times per sample time.

Table 6. Production of Clavulanic Acid with Various Nitrogen Source.

Nitrogen Source	Sample time(h)	Dry Cell Weight (DCW) mg/ml	std dev	[Clavulanic acid] µg/ml	std dev	Specific production µg/mg	std dev
Glutamine	24	2.30	0.140	0.77	0.032	0.335	0.074
	48	4.51	0.014	36.7	0.744	8.14	0.021
	72	4.47	0.027	26.5	0.337	5.93	0.014
	98.5	4.03	0.410	26.5	1.00	6.58	0.108
	122.5	3.57	0.170	31.3	0.336	8.77	0.049
	144	3.31	0.210	2.01	0.021	0.607	0.064
	168	3.04	0.028	7.41	0.176	2.44	0.025
Ammonium Chloride	24	1.25	0.025	1.12	0.042	0.896	0.042
	48	2.51	0.120	26.5	1.01	10.6	0.061
	72	4.20	0.420	7.41	0.281	1.76	0.107
	98.5	3.76	0.170	31.3	1.01	8.32	0.056
	122.5	3.40	0.100	26.5	0.693	7.79	0.039
	144	3.13	0.210	7.41	0.206	2.37	0.073
	168	3.28	0.500	15.0	0.657	4.57	0.159
Asparagine	24	1.46	0.014	0.170	0.007	0.116	0.042
	48	4.51	0.085	56.6	3.30	12.5	0.061
	72	5.28	0.085	36.7	2.22	6.95	0.063
	98.5	4.22	0.120	7.41	0.22	1.76	0.041
	122.5	3.19	0.0071	18.3	0.65	5.75	0.035
	144	3.19	0.190	26.5	1.48	8.31	0.082
	168	2.92	0.070	49.4	4.39	16.9	0.092
Aspartic Acid	24	1.82	0.066	0.000	0.000	0.000	0.000
	48	3.14	0.150	61.6	1.90	19.6	0.057
	72	3.47	0.233	31.3	0.937	9.02	0.074
	98.5	3.20	0.690	42.7	1.26	13.3	0.218
	122.5	2.68	0.017	15.0	0.904	5.60	0.061
	144	2.62	0.112	15.0	0.497	5.73	0.054
	168	2.36	0.095	12.1	0.364	5.13	0.050

Organic nitrogen sources, 2g/L
Inorganic nitrogen sources, 30.4 mM
Dry Cell Weight (DCW) were done in triplicate for each sample time.
Clavulanic acid determinations were done nine times per sample time.

maximum production fluctuated between the nitrogen sources. Again, for the sake of comparison to the cephalosporin nutritional studies in *S. clavuligerus*, asparagine was chosen as the nitrogen source for further studies even though aspartic acid produced slightly higher clavulanic acid levels.

To determine if there was any type of nitrogen regulation, fermentation cultures were carried out with increasing amounts of NH_4Cl added to the production medium already containing 0.2% (w/v) asparagine as the nitrogen source. The production medium was supplemented with 0, 10, 20and 50 mM NH_4Cl. Sampling of the culture filtrates was done in 24 h intervals up to 144 h. Increasing concentrations of NH_4Cl, stimulated growth but clavulanic acid production decreased, indicating some type of regulation (table 7 and figure 15). Without any addition of NH_4Cl, the maximum clavulanic acid production level was 60.5 µg/ml. Clavulanic acid levels dropped while corresponding growth increased with the addition of NH_4Cl. Again the variation in the clavulanic acid levels is quite evident. The numbers are quite low compared to those numbers obtained in the carbon studies. The pattern of production, however, was confirmed by the bioassay.

D. Phosphate Regulation

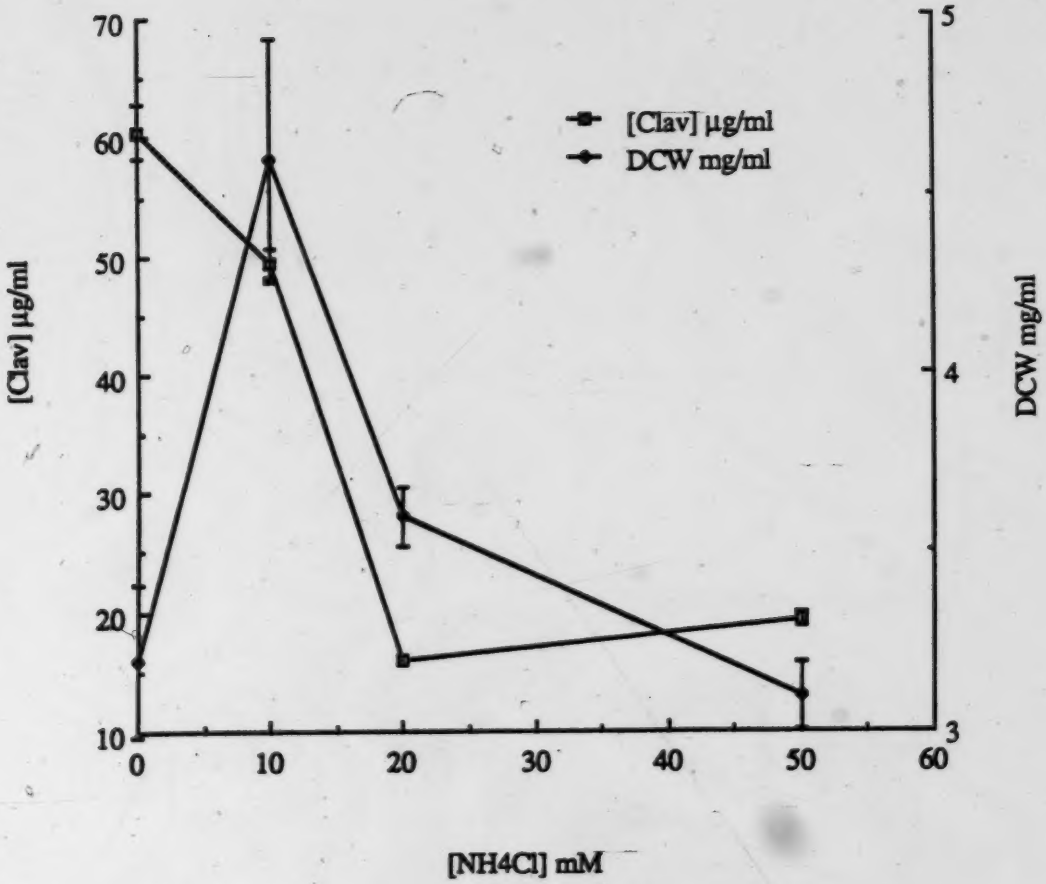
Unlike the situation with carbon and nitrogen sources, there was no investigation of different phosphate sources. The focus was on the effect of phosphate concentration on clavulanic acid production. Cultures were grown in standard production medium with 0, 10, 25, 50 and 75 mM of phosphate. The use of MOPS as the buffer prevented the pH from varying with the addition of different amounts of phosphate. The MOPS buffer was

Table 7. Clavulanic Acid Production with 0,10, 20 & 50 mM Ammonium Chloride Supplements to Production Medium.

[Ammonium Chloride] mM	Sample time(h)	Dry Cell Weight (DCW) mg/ml	std dev	[Clavulanic acid] µg/ml	std dev	Specific Production µg/mg	std dev
0	24	2.40	0.160	21.0	0.372	8.75	0.604
	48	4.80	0.076	37.3	0.663	7.77	0.185
	72	3.60	0.400	15.6	0.722	4.33	0.522
	96	3.20	0.210	60.5	2.25	18.9	1.43
	121	3.10	0.200	22.0	0.918	7.10	0.545
	144	2.50	0.120	21.0	0.973	8.40	0.560
10	24	2.50	0.092	26.9	1.50	10.8	0.718
	48	5.40	0.120	34.5	1.60	6.39	0.328
	72	4.60	0.340	49.4	1.21	10.7	0.836
	96	3.70	0.110	8.69	0.298	2.35	0.107
	121	3.20	0.031	8.69	0.209	2.72	0.070
	144	3.00	0.180	12.6	0.338	4.20	0.276
20	24	2.20	0.100	11.3	0.201	5.14	0.251
	48	5.80	0.025	6.58	0.188	1.13	0.033
	72	5.00	0.100	2.82	0.059	0.564	0.016
	96	3.90	0.310	11.3	0.323	2.90	0.245
	121	3.60	0.081	15.9	0.250	4.42	0.121
	144	3.20	0.062	8.69	0.136	2.72	0.068
50	24	1.90	0.064	1.80	0.131	0.947	0.076
	48	5.10	0.120	2.62	0.095	0.514	0.022
	72	4.80	0.140	5.72	0.283	1.19	0.068
	96	4.50	0.750	2.63	0.107	0.584	0.100
	121	3.10	0.170	3.39	0.142	1.09	0.075
	144	3.10	0.095	19.4	0.582	6.26	0.268

Production medium has 2 g/L asparagine as the nitrogen source.
Dry Cell Weight (DCW) were done in triplicate for each sample time.
Clavulanic acid determinations were done nine times per sample time.

Figure 15. Effect of NH_4Cl Supplementation of Production Medium on Clavulanic Acid Production. The maximum volumetric titre of clavulanic acid produced with increasing amounts of NH_4Cl added to the production medium already supplemented with 0.2 g/100ml asparagine. The carbon source was 1% (w/w) glycerol. The phosphate source was 25mM KH_2PO_4.


70
60
50
40
30
20
10
[Clav] µg/ml
[Clav] µg/ml
DCW mg/ml
5
4
3
DCW mg/ml
0
10
20
30
40
50
60
[NH4Cl] mM

not metabolized by the culture even though it was present in a high concentration (100mM). Sampling of the culture filtrates was done in 24 h intervals up to 144 h. The findings of this study show that clavulanic acid production was affected in a erratic manner by higher phosphate concentrations (table 8). When no phosphate was present in the production medium, there was minimal growth, but clavulanic acid production was relatively high at 15.5µg/ml. Production levels remained the same with 10mM phosphate addition (15.1 µg/ml). The concentration of phosphate which gave optimum levels of clavulanic acid production was 25mM. Maximum production was at 120h with 28.5 µg/ml of clavulanic acid produced. At 50 mM phosphate the volumetric titre dropped significantly lower than at 25 mM (figure 16) to 15.9 µg/ml. Unexpectedly, clavulanic acid production levels rose to 26.4 µg/ml with 75mM phosphate.

Unlike the situation with glycerol and nitrogen where increasing the carbon and nitrogen content decreased clavulanic acid, there does not appear to be any specific pattern to indicate phosphate regulation of clavulanic acid production. Instead of decreasing clavulanic acid production with high amounts of phosphate, it appears to stimulate production (figure 16). When the specific production was examined, the situation was clearer. With the exception of 0 mM phosphate, 25mM phosphate supported the highest specific production titre at 11.2 µg clavulanic acid /mg DCW while the specific production for 50 mM and 75 mM phosphate was respectively, 7.23 and 8.15 µg clavulanic acid / mg DCW. The results indicates that the concentration of phosphate required for the best clavulanic acid production was suboptimal for growth.

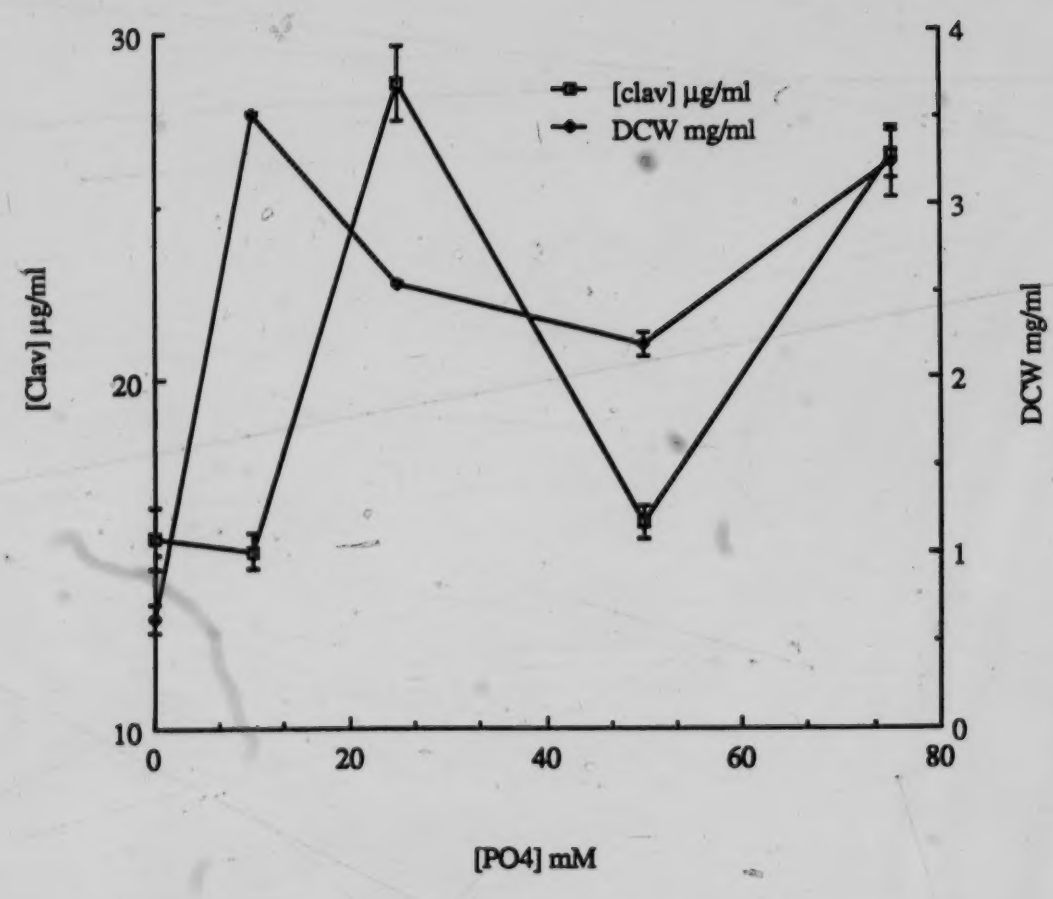
Despite the previously mentioned measures which were taken to standardize inoculum preparation to ensure culture uniformity, clavulanic acid production levels were low in the

Table 8. Production of Clavulanic acid in Response to Phosphate Concentration.

[Phosphate] mM	Sample time(h)	Dry Cell Weight (DCW) mg/ml	std dev	[Clavulanic acid] µg/ml	std dev	Specific Production µg/mg	std dev
0	24	0.720	0.079	3.91	0.234	5.43	0.699
	48	0.657	0.025	15.5	0.898	23.6	1.65
	72	0.673	0.015	9.58	0.422	14.2	0.708
	96	0.640	0.079	14.0	0.609	21.9	2.88
	120	0.897	0.025	13.6	0.648	15.2	0.845
	144	0.875	0.079	13.6	0.455	15.5	1.50
10	24	1.80	0.007	3.53	0.152	1.96	0.075
	48	3.77	0.050	6.10	0.156	1.62	0.073
	72	3.53	0.007	15.1	0.508	4.28	0.147
	96	2.66	0.156	6.25	0.193	2.35	0.153
	120	1.89	0.117	5.38	0.201	2.85	0.229
	144	2.08	0.219	4.83	0.254	2.32	0.324
25	24	1.79	0.665	7.36	0.489	4.11	2.83
	48	3.64	0.072	10.3	0.256	2.83	0.098
	72	3.58	0.052	13.3	0.359	3.72	0.118
	96	3.10	0.093	14.3	0.608	4.61	0.257
	120	2.55	0.007	28.5	1.10	11.2	0.456
	144	1.89	0.099	15.0	0.266	7.94	0.472
50	24	1.64	0.007	5.53	0.140	3.37	0.085
	48	4.02	0.208	12.7	0.558	3.16	0.231
	72	3.95	0.100	9.51	0.674	2.41	0.204
	96	3.38	0.135	13.3	0.619	3.93	0.248
	120	2.20	0.071	15.9	0.505	7.23	0.322
	144	2.85	0.047	11.0	0.378	3.86	0.154
75	24	1.30	0.015	5.31	0.117	4.08	0.104
	48	3.24	0.208	26.4	0.692	8.15	0.550
	72	4.52	0.110	19.9	0.553	4.40	0.159
	96	3.90	0.064	22.8	0.727	5.85	0.222
	120	3.50	0.007	18.6	0.534	5.31	0.185
	144	3.34	0.141	23.3	0.884	6.98	0.396

Dry Cell Weight (DCW) were done in triplicate for each sample time.
Clavulanic acid determinations were done nine times per sample time.

Figure 16. Effect of Phosphate Concentration on Clavulanic Acid Production. The maximum volumetric titre of clavulanic acid produced with increasing amounts of KH_2PO_4 added to the production medium. The carbon source was 1% (w/w) glycerol. The nitrogen source 0.2 g/100ml asparagine.


[clav] µg/ml
DCW mg/ml
[Clav] µg/ml
DCW mg/ml
[PO4] mM
30
20
10
4
3
2
1
0
0
20
40
60
80

fermentation runs carried out to investigate the effects of phosphate concentration. Since the standard production medium contained 25 mM phosphate, the 25 mM culture in these fermentation runs were expected to show levels of clavulanic acid production similar to those seen in the studies on carbon and nitrogen sources. Although the reason for the low levels of clavulanic acid production is unknown, phosphate concentration did affect clavulanic acid production.

V. DISCUSSION

The results describe the development of analytical procedures for the quantitation of clavulanic acid production in *S. clavuligerus*. Once an accurate and reliable assay system for measuring clavulanic acid was obtained, the effect of carbon, nitrogen and phosphate sources on clavulanic acid production was investigated. The studies showed that clavulanic acid production was affected by the amount of carbon and nitrogen available. The effect of the amount of phosphate on clavulanic acid production was less clear.

V.1. Clavulanic Acid Analyses

A. Biological assay (Bioassay) of Clavulanic acid.

The biological assay was determined to be unsuitable as a quantitative analysis system for clavulanic acid in this study. The method described by Reading and Cole (1977), although it was convenient and rapid method of checking the culture filtrates, had several flaws that made it unreliable. One problem was the sensitivity of the bioassay. As indicated by the results, small changes in the zone size correlated to large changes in the amount of clavulanic acid detected. The minimum zone size of 6.35 mm made detection of amounts of clavulanic acid less than 0.05 μg difficult. The physiological state of the indicator organism also affected the zone size (data not shown). With a young indicator culture as inoculum, the resulting zone sizes of clavulanic acid standards and culture filtrates were small. With older indicator cultures, the zone sizes were much larger. Standardized indicator inoculum alleviated some of the variability. While unreliable for quantitative detection, the bioassay was a rapid method of qualitatively determining the presence of clavulanic acid.

B. Chemical Assay

The chemical assay described by Bird *et al.* (1982) and Foulstone and Reading (1982) was investigated as an alternative to the bioassay. The imidazole-derivatized clavulanic acid standards produced a linear response. The ultra violet (uv) scans of these standards showed a single peak with an absorbance maximum at 311 nm and the scans of the controls showed negligible absorbance. This corresponded to the results obtained by Foulstone and Reading (1982).

However, discrepancies in the chemical assay became evident with the analysis of culture filtrates. The imidazole treated culture filtrate did not have the expected absorbance peak at 311 nm but rather, a plateau. When compared to the imidazole treated culture filtrates, the scans of the untreated culture filtrates had a high background absorbance. It appeared that the culture filtrate itself was masking the derivation process of the imidazole reagent.

To determine the nature of the interference, various controls were included. The imidazole reagent, uninoculated production medium and uninoculated production medium when reacted with imidazole, had low uv absorbances. Similarly, uninoculated production medium supplemented with standard clavulanic acid, showed little uv absorbance. When this sample was treated with imidazole, there was a peak of absorbance at 311nm as expected. This result showed that the interfering material was not in the uninoculated production medium.

However, when a culture filtrate was supplemented with the same amount of clavulanic acid, and reacted with imidazole, the absorbance was lower than the clavulanic

acid standard alone. A higher absorbance was expected since there was clavulanic acid initially present in the filtrate (indicated by the bioassay).

Changing the type of carbon and nitrogen sources in the production medium also interfered with the assay. Some of the untreated culture filtrates gave higher absorbances than the derivatized sample.

These problems in the spectrophotometric chemical assay indicated that the interfering material was present in the culture filtrate after fermentation. Attempts to resolve the problem by filtration and extraction were unsuccessful (data not shown). Because of the nature of the nutritional studies, this assay was unsuitable for quantitating clavulanic acid from culture filtrates.

C. High Pressure Liquid Chromatography (HPLC) analysis.

Analysis of clavulanic acid by HPLC was also not satisfactory. Initial analysis of clavulanic acid in the culture filtrates was hampered by the interference caused by media components. The changes in the carbon and nitrogen sources, buffer and fermentation products made it difficult to resolve the clavulanic acid peak from interfering materials. Clavulanic acid standards in uninoculated production medium or H_2O, were only marginally resolved. Culture filtrates supplemented with clavulanic acid gave peak areas which could not be measured reliably because of interfering peaks.

The addition of PIC A reagent (Waters), an ion pairing reagent, to the mobile phase initially improved the resolution and separation of clavulanic acid standard in H_2O. Resolution was also improved in the culture filtrates. Closer inspection of the data revealed that the values obtained from HPLC did not correlate to the numbers indicated by

the bioassay, the values were 100 fold higher than the semi-quantitative bioassay. In addition, it was determined that the MOPS buffer in the media, was complexing with the Pic A reagent resulting in the formation of a peak with the same retention time as the clavulanic acid standards. An alternative HPLC method was investigated since MOPS was the essential buffering component in the production medium and could not be replaced.

Pre-column derivatization of the clavulanic acid with imidazole, as described by Foulstone and Reading (1982), improved detection and resolution of clavulanic acid from the other components of the medium. The imidazole-derivatized clavulanic acid product was resolved with no interference from underivatized clavulanic acid. Linearity was obtained for plots of peak area versus concentration.

The method of Foulstone and Reading (1982) was the most reliable HPLC system used but it still suffered from interference problems when the nutrient sources were changed. Chromatograms of the untreated culture filtrate showed peaks being resolved at retention times close to the imidazole treated culture filtrate. These peaks contribute to the area of the derivatized peak and made quantitation very difficult especially at low concentration of clavulanic acid. With this development, the HPLC was no longer used as an assay method.

D. Enzyme assays.

The fourth analytical method investigated for the quantitation of clavulanic acid was the enzyme based ß-lactamase inhibition assay. Two different ß-lactam substrates, PADAC and penicillin G, were assessed.

The coloured cephalosporin, PADAC, has been used clinically for screening for ß-lactamase producing organisms. It has also been described as a rapid method of detecting ß-lactamase inhibitors from crude culture fermentation broths (Schindler and Huber, 1980 and Jones *et al.*, 1982). The advantage of using the PADAC substrate over penicillin G was that the reaction can be measured at the visible end of the wavelength spectrum.

Addition of clavulanic acid did inhibit the rate of PADAC degradation by the ß-lactamase enzyme. The major flaw with this assay became evident when the specificity of the assay was investigated. Cephamycin C, which is a product of the fermentation, was found to inhibit the enzyme although not to the same degree as clavulanic acid. As mentioned previously, the possible reason for the result was that cephamycin C, being similar in structure to PADAC, could have been competing with the coloured cephalosporin. Although it is quite resistant to degradation by ß-lactamase, cephamycin C may inhibit the enzyme and thereby reduce its ability to degrade PADAC. To circumvent the interference problem, attempts were made to use a mutant that did not produce penicillins and cephalosporins. While unable to produce ß-lactam antibiotics, the mutant strain was still able to produce clavulanic acid. Bioassays of the NTG # 12 culture filtrate indicated production of clavulanic acid when NTG # 12 was grown in a complex medium. Preliminary tests with PADAC confirmed this but when the mutant was cultured in the defined nutritional production medium, no growth was observed. The auxotrophic nature of the mutant made it unsuitable for further nutritional studies.

The second enzyme assay that was used to quantitate clavulanic acid was the method

described by Reading and Hepburn (1979), and Gutman *et al.* (1985b) using penicillin G as the substrate. This method monitors the degradation of penicillin G at A_{240}nm. Quantitation of clavulanic acid was done by interpolating plots of the percentage of rate inhibition to the amount of clavulanic acid. The plot of percentage of rate inhibition versus the amount of clavulanic acid gives a non linear relationship. The accuracy was dependent on the curvature of the plot. To improve interpolation, the amount of clavulanic acid was differentiated before being plotted. Plots of the percentage of rate inhibition versus the cubed root of the amount of clavulanic acid produced linear relationships. This is different from the situation reported by Reading and Hepburn (1979). In their study, a linear relationship was obtained by plotting the percentage of rate inhibition against the amount of clavulanic acid. Their enzyme assays were conducted with the *S. aureus* ß-lactamase. The inactivation of the *S. aureus* ß-lactamase by clavulanic acid is a stoichiometric, one-to-one relationship. The non linear relationship of the curve obtained with the Difco penicillinase (from *Bacillus cereus*), is the result of the more complex kinetics of the particular enzyme reacting with clavulanic acid. Much more clavulanic acid is required to inactivate this enzyme. Gutman *et al.* (1985b) found similar results using ß-lactamase from *B. cereus* and the same procedure as this study. Instead of trying to linearize the standard curve, they left it as a non linear relationship. By linearizing the standard curve, the values obtained in the subsequent nutritional studies approached the reported literature values.

Unlike the PADAC assay, there was no interference by cephamycin C when penicillin G was used as the substrate for the ß-lactamase assay. However, there was other

interference from the culture filtrate. Some filtrates that tested negative for clavulanic acid by the bioassay nonetheless inhibited the ß-lactamase indicating the presence of clavulanic acid. Separate studies have since shown that *S. clavuligerus* produces an extracellular proteinaceous ß-lactamase inhibitor in addition to clavulanic acid. This interference problem, which gave false positive results, was alleviated by the addition of equal volumes of methanol to the culture filtrates. Standard solutions of clavulanic acid were not affected by the methanol addition, nor was the ß-lactamase enzyme.

2. NUTRITIONAL STUDIES

The study of clavulanic acid production in *S. clavuligerus* was based on the parallel experiments by Aharonowitz and Demain on cephalosporin production in the same organism (Aharonowitz and Demain, 1977, 1978, 1979). In their original studies, Aharonowitz and Demain investigated the effect of carbon sources, nitrogen sources and phosphate concentration on cephalosporin production. These same criteria have been applied to clavulanic acid production in *S. clavuligerus*.

A. CARBON SOURCES

In studying the production of cephalosporin in *S. clavuligerus*, Aharonowitz and Demain found the best carbon sources for production were starch, glycerol and maltose. They also determined that organic acids such as succinate, fumarate, and α-ketoglutarate were suitable carbon sources (Aharonowitz and Demain, 1978). With cephalosporin production, the poorer carbon sources gave low volumetric production of the antibiotic but higher specific production of cephalosporin (µg/mg DCW) and shifted the fermentation to be more closely associated with growth.

The results of this study showed that the carbon sources most suitable for growth and the production of clavulanic acid in *S. clavuligerus*, were maltose, glycerol, starch and dextrin. This finding is similar to the cephalosporin results obtained by Aharonowitz and Demain. Unlike the situation with cephalosporin production, there was no production of clavulanic acid with organic acids such as succinate and lactate. α-ketoglutarate did support a low level of growth and production but not as good as the preferred carbon sources (data not shown).

Both sucrose and glucose have been mentioned in the literature as being suitable carbon sources for growth of *S. clavuligerus* (Cole *et al.*, 1985). The results from this study and from Aharonowitz and Demain do not support this finding. Rather they support the evidence presented by other groups, that sucrose and glucose are non utilizable carbon sources (Higgins and Kastner, 1971 and Arai *et al.*, 1975). As with the situation found in cephalosporin production, the poorer carbon sources lead to higher specific production (μg/mg DCW) of clavulanic acid (low volumetric production) and shifted the fermentation to be more closely associated with growth.

The growth response and clavulanic acid production levels observed with maltose, starch and dextrin, as the sole carbon source were similar. Maximum production levels ranging from 129-174 μg/ml of clavulanic acid were produced with each of these substrates. Production was slightly lower with glycerol as the carbon source (84.7-169 μg/ml). As mentioned earlier, glycerol was still chosen as the carbon source for further studies to facilitate comparison with the studies of Aharonowitz and Demain and help to clarify the relationship between clavulanic acid production and cephalosporin production

in *S. clavuligerus*.

Other investigators used non defined carbon sources or multiple carbon sources (Cole *et al.*, 1985 and Romero *et al.*, 1984). The media described in the U.S. patent by Cole *et al.*, were comprised of complex nutrients such as peptone, malt extracts and soybean meal which served as both the carbon and nitrogen sources. While these media did give good production of clavulanic acid, they did not give much information as to the specific carbon and nitrogen sources which promote clavulanic acid production. Few defined nutritional studies on clavulanic acid production have been carried out. In one such study by Romero *et al.* (1984), the carbon source consisted of a mixture of sucrose and glycerol, with glutamic acid and proline as the nitrogen source. They found that clavulanic acid production coincided with the rapid utilization of glutamic acid. The sucrose was utilized at a slower rate than glycerol. When *S. clavuligerus* was grown in production medium without glycerol, there was no production of clavulanic acid. The maximal rate of clavulanic acid production was obtained when 110mM (1% w/v) glycerol was initially present. Concentrations above this level reduced the production of clavulanic acid (Romero *et al.*, 1984). In another study by Ho in 1985, using conditions very similar to Romero *et al.*, high levels of clavulanic acid production were achieved in a glycerol based defined medium but in this case the level of glycerol was much higher at 3.5% w/v (380mM).

These previous findings that glycerol is a good carbon source for clavulanic acid production, correlate well with the present study. However, Romero *et al.* suggest that glycerol is a direct precursor of clavulanic acid and was therefore an essential component

of the growth medium for clavulanic acid production. The good levels of clavulanic acid production found in this study with starch, maltose or dextrin as carbon sources clearly do not support this hypothesis.

In this study, fluctuations in the levels of clavulanic acid production were frequently observed between fermentations. The values do not always approach the reported literature values (200-500μg/ml) (Cole *et al.*, 1985). With 1% (w/v) glycerol, the maximum production levels ranged from 84.7 to 169 μg/ml. The major factor affecting this probably was the physiological state of the culture. Although the glycerol spore stocks were germinated in the same manner for each fermentation, and the inoculation procedure was kept constant, there was always a fluctuation with the growth and production levels. Other methods such as a spore count with a counting chamber would have given a more accurate number but these methods were logistically impractical. Counting germinated spores would be difficult due to the filamentous nature of the bacterium in use.

To minimize the variation in the results, the inoculum size was standardized as much as possible (as described in the Materials and Methods). Everything was done in triplicate. Initially the culture filtrates were pooled and assayed in triplicate. This was changed so that each culture filtrate was analyzed in triplicate. The numbers obtained from this inoculum procedure still varied but the clavulanic acid production pattern appeared to stabilize.

B. GLYCEROL

Possible carbon catabolite regulation on clavulanic acid production was investigated

by growing the cultures in production medium with different amounts of glycerol. Initial HPLC results had indicated that in the absence of glycerol, there was almost no clavulanic acid production or growth. The first detectable levels were obtained with 0.5 % (w/v) glycerol as the carbon source. At concentrations greater than 1% glycerol, clavulanic acid levels decreased. This is consistent with the results of Romero *et al.* (1984) and Aharonowitz and Demain (1978). Filtrates analyzed with the penicillin G enzyme assay generally support this pattern and so a glycerol content of 1% was used in subsequent studies. The difference in the production levels of clavulanic acid from literature values (200-500 μg/ml Cole *et al.*, 1985 and Ho, 1985) could be due to the use of one carbon source in this study instead of a combination of carbon sources that could enhancing the production. The only carbon source present is glycerol. It may be argued that the nitrogen source, asparagine, could be a possible carbon source as well but the small amount of carbon present in the asparagine made no significant impact on the production of clavulanic acid. When asparagine was tested as both the nitrogen and carbon source, the amount of clavulanic acid produced was negligible.

C. NITROGEN REGULATION

There have been many studies which demonstrate that the type of nitrogen source present can regulate antibiotic production. One such example is the regulation of cephalosporin production in *S. clavuligerus* by various nitrogen sources. Aharonowitz and Demain (1979) and Brana *et al.* (1985) determined that as nitrogen sources, certain amino acids supported good levels of both growth and production of cephalosporin while NH_4Cl supported growth but gave decreased levels of antibiotic production. The best

amino acids for cephalosporin production in *S. clavuligerus* were asparagine and glutamine with arginine the next best. When NH_4Cl was added to asparagine supplemented production medium, the growth rate was not affected but cephalosporin production was greatly reduced. Cephalosporin production was reduced by 75%. From their studies on cephalosporin production, Aharonowitz and Demain determined that the addition of low levels of NH_4Cl to medium already containing asparagine stimulated antibiotic production and growth. Addition of concentrations of NH_4Cl greater than 10mM, lowered the antibiotic yields while the biomass increased. From this, they concluded that the fermentation, in the absence of added NH_4Cl, was nitrogen limited. When nitrogen in the form of NH_4Cl was in excess (above 10mM), production fell drastically (Aharonowitz and Demain, 1979).

In this study, the initial screening of nitrogen sources showed that urea and $NaNO_3$ were unsuitable as nitrogen sources. A maximum growth of 0.9 mg/ml dry cell weight (DCW) was reached with 20 mM urea while literature reported growth to reach a maximum of 2.5 mg/ml DCW (Aharonowitz and Demain, 1979). $NaNO_3$ supported no growth and low production of clavulanic acid. Good clavulanic acid production was obtained when the nitrogen source was aspartic acid, asparagine, or glutamine. In an initial study, NH_4Cl supported high clavulanic acid production levels but this result was not observed in subsequent fermentations, where the production of clavulanic acid was poor.

When the effect of addition of NH_4Cl to medium already containing asparagine on the production of clavulanic acid was investigated, there was no stimulation of production with the addition of 10mM NH_4Cl. Maximum clavulanic acid production was achieved without any addition of NH_4Cl. Increasing the concentrations of NH_4Cl stimulated growth but clavulanic acid production decreased indicating regulation. Production dropped by 70.6%. The corresponding growth rose 33.7%. This result show that the negative effect of ammonium salts on clavulanic acid production in *S. clavuligerus* is similar to the effect on cephalosporin production in *S. clavuligerus* as reported by Aharonowitz and Demain (1979) and Brana *et al.* (1985) but no evidence of stimulation by low levels of NH_4Cl was seen.

Nitrogen repression of clavulanic acid production in *S. clavuligerus* has also been reported by Romero *et al.*, (1984). The nitrogen sources used in that study were proline and glutamic acid. Glutamic acid was found to be rapidly utilized. Coinciding with the glutamic acid depletion was the onset of clavulanic acid production. Addition of more glutamate or NH_4Cl to the production medium reduced production of clavulanic acid. The additional nitrogen also delayed the onset of clavulanic acid production until the remaining glutamic acid was utilized. This indicated to them that there was some nitrogen catabolite regulation on the biosynthesis of clavulanic acid. Although Romero *et al.* used different conditions, they do show that clavulanic acid production is suppressed by additional nitrogen.

D. PHOSPHATE REGULATION

Aharonowitz and Demain (1977) found that cephalosporin production in *S. clavuligerus* is affected by phosphate in the same manner as most secondary metabolism. The best production yields are obtained at phosphate concentrations that are sub optimal for vegetative growth. The low concentrations of phosphate were insufficient to provide the necessary buffering action for the growth medium. To stabilize the pH, Aharonowitz and Demain increased the phosphate concentration. At higher concentration of phosphate, growth was stimulated and the pH was stabilized. However, at high concentrations of phosphate, cephalosporin production was inhibited. To circumvent this problem, they used MOPS as the non metabolizable buffer, and looked at the effect of phosphate on cephalosporin production in a buffered system. At low concentrations, the growth and antibiotic production was low. When the concentration was increased to 10 mM, growth was no longer limited and the production of cephalosporin was increased. Both the rate and extent of antibiotic production was found to be inhibited at phosphate concentration greater than 25 mM (75mM-100mM), but growth was supported (Aharonowitz and Demain, 1977). The inhibition of production was progressive, up to 85% lower than at the optimum concentration. Lubbe *et al.* (1985) duplicated these results although the reduction of cephalosporin production was less drastic. The specific production decreased by 50-70%.

When the effect of phosphate was examined in this study, the results indicated that there was not a drastic effect of phosphate concentration on clavulanic acid production. These results are difficult to interpret because of an overall decrease in clavulanic acid production levels. The maximum clavulanic acid levels dropped from approximately 150

µg/ml to about 30 µg/ml. This lower clavulanic acid production level was observed even though the production medium remained the same as had been used throughout the study. Even with the lower production levels, it was still possible to determine the effect of phosphate on clavulanic acid production.

With no phosphate present in the production medium, there was minimal growth. Production was relatively high. Increasing the phosphate concentration to 10mM decreased the clavulanic acid production slightly. The concentration of phosphate required for optimum clavulanic acid production was 25mM. At higher concentrations of phosphate (50 and 75 mM), the volumetric and specific production titre of clavulanic acid was not significantly lower than at 25 mM. The decrease in production was not as drastic as the situation described by Aharonowitz and Demain (1977). With 75 mM phosphate, the optimum volumetric titre was 26.4 µg/ml versus 28.5 µg/ml at 25 mM, a reduction of 7.37 %. The maximum specific production titre dropped only by 27.2 % (11.2 to 8.15 µg/mg dcw).

The effect of phosphate concentration on clavulanic acid production in *S. clavuligerus* has also been reported by Romero *et al.* (1984). They determined that there was a concentration dependent inhibitory effect on clavulanic acid production in resting cells. In a MOPS buffered system, addition of phosphate up to 25mM stimulated clavulanic acid production. Higher concentration of phosphate inhibited clavulanic acid production.

VI. Conclusion

Summing up, this study shows that the most reliable method for quantitating clavulanic acid from fermentation broths is the ß-lactamase enzyme assay with penicillin

G as the substrate. The assay is quick and is not affected by the change in the production medium constituents.

The study of clavulanic acid production in *S. clavuligerus* indicate similarities and differences with the parallel experiments done by Aharonowitz and Demain on cephalosporin production in the same organism. The similarities with cephalosporin production are: the use of glycerol, starch and maltose as carbon source for optimal clavulanic acid production, the carbon catabolite effect with increased amounts of glycerol, the nitrogen repression on production and the adverse effect of increasing phosphate concentration on production. The differences with cephalosporin production are: the failure to observe stimulation of clavulanic acid production with slight increases in NH_4Cl concentration, and the less drastic reduction of clavulanic acid production with increased amounts of nitrogen and phosphate. This study also showed the difficulty in obtaining reproducible levels of clavulanic acid production, probably due to the complex growth characteristics of the organism.

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